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SETE ESPÉCIES DE CONNARACEAE: COMPOSIÇÃO QUÍMICA E POTENCIAL EFEITO BIOLÓGICO PARA O TRATAMENTO DO DIABETES

LUÍS FERNANDO NUNES ALVES PAIM

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Tese apresentada ao Programa de Pós-graduação em Biotecnologia da Universidade de Caxias do Sul, como parte dos requisitos para a obtenção de grau de Doutor em Biotecnologia

Orientador: Prof. Dr. Sidnei Moura e Silva

Co-Orientadora: Profa. Drª Mirian Salvador

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“É assustador pensar que tudo o que hoje aprendi e produzi, com o auxílio de generosos outros, será num futuro nem tão distante algo diferente ou mesmo ultrapassado. Apego-me apenas ao fato de que mesmo hoje disto sabendo não me eximi em apropriar-me ou pelo menos convencer-me de que assim fosse. Quando escolhi a ciência, eu não sabia que ela é a mais fiel das criações do processo evolutivo. Foi no aprofundamento do estudo científico que descobri que tudo no universo evolui e muda constantemente. Mesmo assim, jamais pensei em desistir ou fazer coisa diferente; e se outra vez me fosse ofertada uma escolha, novamente escolheria a ciência. Isto por que, por mais mutável e imprevisível que seja, a ciência, em sua única exceção, mantém a libertação humana como a sua principal característica.” (O autor).

ÍNDICE

LISTA DE FIGURAS

Lista de siglas e abreviaturas

4-NPGP - 4-nitrofenil α-D-glucopiranosídeo

AGEs - *Advanced Glycation End Products*

CBAF - Componente Básico da Assistência Farmacêutica

CL/EM - Cromatografia líquida associada a espectrometria de massas

CLAE - Cromatografia a liquido de alta eficiência

CNPG3 - 2-cloro-4-nitrofenil α-D-maltotriosídeo

Da - Daltons

DM – Diabetes *Mellitus*

DMEM - *Dulbecco's Modified Eagle Medium*

DMSO - Dimetil sulfóxido

DPPH - 2,2-difenil-1-picril-hidrazil

EA.hy926 - *Human umbilical vein cell line*

EM - Espectrometria de massas

EMAR - Espectrometria de massas de alta resolução

FRAP - *Ferric Reducing Antioxidant Power*

FT-ICR -*Transform ion cyclotron resonance*

GNPS - *Global Natural Products Social*

MS/MS - Espectrometria de massas em tandem

MTT - Brometo de 3-[4,5-dimetiltiazol 2-il]-2,5-difeniltetrazólio

OMS - Organização Mundial da Saúde

OT - *Ion trap*

Q-TOF - Quadrupolo – Tempo de voo

Q-trap - Quadrupolo – *Trap*

ROS - *Reactive oxygen species*

SBF - Soro fetal bovino

SMILE - *Simplified Molecular Input Line Entry Specification*

STZ – Estreptozotocina

SUS - Sistema Único de Saúde

TOF- *Time-of-flight*

RESUMO

Connaraceae é uma família de plantas com alto potencial farmacológico, ao nível mundial constatamos que 39 táxons estão associados ao uso tradicional ou possuem relatos científicos acerca da utilização em pesquisas de atividades farmacológicas. Mundialmente, cerca de 200 espécies de Connaraceae foram descritas e entre essas apenas 10 têm sua composição química conhecida. No Brasil essa família de plantas pode ser encontrada nos biomas Caatinga, Cerrado, Floresta Amazônica e Mata Atlântica. O Cerrado e a Mata Atlântica são considerados biomas altamente impactados (*hotspots*) pela ação antrópica e nestes cerca de metade dos táxons têm seu habitat. Portanto as espécies de Connaraceae endêmicas destas áreas estão mais susceptíveis a extinção. Entre todas as espécies de Connaraceae *Agelaea pentagyna, Cnestis ferruginea, Connarus suberosus* e *Rourea minor* têm o mais avançado estágio de desenvolvimento de pesquisas e, portanto, estão mais próximas a dar origem a medicamentos úteis a humanidade. Na utilização etnobotânica as diversas formas de preparação incluem entre outros métodos a decocção, infusão e maceração. Assim, propomos estudar a composição química sobre a ótica qualitativa em quatro espécies do gênero *Connarus*, incluindo *C. blanchetii, C. nodosus, C. regnellii* e *C. suberosus.* Utilizamos a cromatografia líquida associada a espectrometria de massas (CL/EM) e através do estudo dos dados produzidos utilizando plataformas de bioinformática identificamos nessa fase 23 compostos a partir dessas espécies. Do ponto de vista qualitativo a maioria dos metabólitos pode ser identificada frente aos três métodos. Entre os principais compostos associados a essas espécies estão a quercetina, miricetina, guaijaverina, hiperina, miricetrina e querciturona sobre os quais conduzimos uma breve revisão acerca do potencial farmacológico. Na sequência utilizando apenas os extratos obtidos por maceração de *C. blanchetii, C. regnellii, C. suberosus* e *R. glazioui* conduzimos um fracionamento orgânico e evidenciamos que as frações mais polares, acetato de etila e n-butanólica possuem o melhor perfil quantitativo de metabólitos secundários de interesse as atividades que propomos. Assim, buscamos nas frações acetato de etila e n-butanólica estudar o potencial antioxidante. Compostos de origem vegetal têm sido estudados como alternativas para minimizar o estresse oxidativo em pacientes portadores de diabetes, visto que essa é um dos eventos associados a complicações desta doença. Em adição ao potencial antioxidante estudamos o efeito inibitório dos compostos associados a estes extratos em reduzir a glicação proteica pelas vias oxidativa e não oxidativa. Os metabólitos secundários foram estudados via CL/EM e identificados através das plataformas de bioinformática. Os resultados dessa fase apontaram para um melhor desempenho antioxidante para as frações n-butalólica de *C. blanchetii, C. regnellii* e *C. suberosus* e acetato de etila/n-butalólica de *R. glazioui.* Na avaliação da atividade antiglicante para a via oxidativa *R. glazioui* fração de acetato etila e *C. suberosus* fração n-butanólica apresentam o melhor desempenho. Para a via não oxidativa *R. glazioui* fração de acetato etila *C. blanchetii* fração n-butanólica mostraram-se as mais promissoras. Entre *C. blanchetii, C. regnellii, C. suberosus* e *R. glazioui* 29 metabólitos foram identificados. As atividades antioxidantes e antiglicantes observadas podem ser relacionadas com vários compostos presentes nessas espécies entre eles os flavonoides apigenina, quercetina, miricetina e seus derivados. Com isso propomos que essas espécies tem potencial para dar origem a medicamentos úteis no controle das complicações do diabetes. Sequencialmente, conduzimos uma análise da atividade inibitória dos extratos de *C. blanchetii, C. nodosus, C. regnellii, C. suberosus, R. cuspidata, R. glazioui* e *R. induta* frente as enzimas α-amilase e α-glicosidase. Os resultados obtidos apontam que todas essas espécies se apresentam pouco ativas frente a essas duas enzimas relacionadas com o metabolismo de carboidratos. Frente a essas sete espécies 34 metabólitos secundários foram identificados. Por fim propomos estudar os efeitos tóxicos das frações n-butanólicas de *C. suberosus* e *R. cuspidata* e das frações acetato etila e n-butanólica de *R. glazioui* bem como a habilidade dessas frações em reverter a citotoxicidade celular em

células endoteliais. Observamos que nenhuma das espécies apresenta-se tóxica para essas células e que quanto testadas nas concentrações de 10 e 25 µg/mL são capazes de reverter os danos celulares causados pela alta concentração de glicose. Portanto, concluímos que essas espécies de Connaraceae podem ser úteis no controle de muitas das complicações do diabetes. Adicionamos 4 novas espécies dentre elas *C. blanchetii, C. nodosus, C. regnellii* e *R. glazioui* ao rol dos táxons de Connaraceae com constituição química estabelecida e revelamos o potencial farmacológico dessas espécies como promissoras no controle das complicações do Diabetes a comunidade científica.

ABSTRACT

Connaraceae is a family of plants with high pharmacological potential, worldwide we found that 39 taxa are associated with traditional use or have scientific reports about their use in research on pharmacological activities. Worldwide, about 200 species of Connaraceae have been described and among these only 10 have their chemical composition known. In Brazil, this family of plants can be found in the Caatinga, Cerrado, Amazon Rainforest and Atlantic Forest biomes. The Cerrado and the Atlantic Forest are considered biomes highly impacted (hotspots) by anthropic action and in these, about half of the taxa have their habitat. Therefore, Connaraceae species endemic to these areas are more susceptible to extinction. Among all Connaraceae species, *Agelaea pentagyna*, *Cnestis ferruginea*, *Connarus suberosus* and *Roura minor* have the most advanced stage of research development and, therefore, are closest to giving rise to useful medicines for mankind. In ethnobotanical use, the various forms of preparation include, among other methods, decoction, infusion and maceration. Thus, we propose to study the chemical composition under qualitative optics in four species of the genus *Connarus*, including *C. blanchetii, C. nodosus, C. regnellii* and *C. suberosus.* We used liquid chromatography associated with mass spectrometry (LC/MS) and by studying the data produced using bioinformatics platforms, we identified 23 compounds from these species in this phase. From a qualitative point of view, most metabolites can be identified using the three methods. Among the main compounds associated with these species are quercetin, myricetin, guaijaverin, hyperin, myricetrin and querciturone, on which we conducted a brief review of their pharmacological potential. Then, using only the extracts obtained by maceration of *C. blanchetii, C. regnellii, C. suberosus* and *R. glazioui*, we conducted an organic fractionation and showed that the more polar fractions, ethyl acetate and n-butanol, have the best quantitative profile of secondary metabolites of interest to the activities we propose. Thus, we sought to study the antioxidant potential in the ethyl acetate and n-butyl acetate fractions. Plant-derived compounds have been studied as alternatives to minimize oxidative stress in patients with diabetes, as this is one of the events associated with complications from this disease. In addition to the antioxidant potential, we studied the inhibitory effect of compounds associated with these extracts in reducing protein glycation through oxidative and non-oxidative pathways. Secondary metabolites were studied via LC/MS and identified through bioinformatics platforms. The results of this phase pointed to a better antioxidant performance for the n-butanol fractions of *C. blanchetii, C. regnellii* and *C. suberosus* and ethyl acetate/n-butanol fractions of *R. glazioui*. In evaluating the antiglycant activity for the oxidative pathway, R. glazioui ethyl acetate fraction and C. suberosus n-butanolic fraction showed the best performance. For the non-oxidative pathway R. glazioui ethyl acetate fraction C. blanchetii n-butanolic fraction were the most promising. Among *C. blanchetii, C. regnellii, C. suberosus* and *R. glazioui* 29 were identified. The antioxidant and antiglycant activities observed can be related to several of the compounds present in these species, including the flavonoids apigenin, quercetin, myricetin and their derivatives. Thus, we propose that these species have the potential to give rise to drugs that are useful in controlling the complications of diabetes. Sequentially, we conducted an analysis of the inhibitory activity of *C. blanchetii, C. nodosus, C. regnellii, C. suberosus, R. cuspidata, R. glazioui* and *R. induta* extracts against α-amylase and α-glucosidase enzymes. The results obtained show that all these species are not very active against these two enzymes related to carbohydrate metabolism. Against these seven species 34 secondary metabolites were identified. Finally, we propose to study the toxic effects of the n-butanolic fractions of *C. suberosus* and *R. cuspidata* and the ethyl acetate and n-butanolic fractions of *R. glazioui*, as well as the ability of these fractions to reverse cell cytotoxicity in endothelial cells. We observed that none of the species is toxic to these cells and that when tested at concentrations of 10 and 25 µg/mL, they are able to reverse the cell damage caused by the high concentration of glucose.

Therefore, we conclude that these Connaraceae species may be useful in controlling many of the complications of diabetes. We added 4 new species among them *C. blanchetii*, *C. nodosus*, *C. regnellii* and *R. glazioui* to the list of Connaraceae taxa with established chemical constitution and revealed the pharmacological potential of these species as promising in the control of diabetes complications to the scientific community.

1 INTRODUÇÃO

O Brasil é um país megadiverso que contém cerca de um quinto de todas as espécies vivas deste planeta e neste contexto considerando apenas a diversidade vegetal, é estimado que o país possua uma quantidade superior a 41 mil espécies (MARTINELLI; MORAES, 2013) destes 33 mil já foram documentadas e identificadas (FORZZA *et al.*, 2010a, 2010b). Desta forma, há um potencial farmacológico dessa biodiversidade a ser explorado, pois muitas destas espécies podem estar produzindo metabólitos secundários úteis ao homem, os quais podem ser utilizados diretamente e/ou servirem de protótipos para a obtenção de compostos sintéticos (VILLAS BÔAS; GADELHA, 2007). Embora carecendo de pesquisas mais recentes, uma pesquisa de 2008 mostrou que menos de 26% do total de medicamentos fitoterápicos registrados no Brasil advinham de espécies nativas da América do Sul (CARVALHO *et al.*, 2008). Deste modo, considerando o potencial biofarmacológico com o número de fármacos desenvolvidos, é notório o baixo aproveitamento da biodiversidade brasileira. Assim, somos ainda carentes de pesquisas capazes de desvendar este potencial, o qual poderia gerar uma vantagem competitiva frente ao mercado global destes bens (VILLAS BÔAS; GADELHA, 2007). Paradoxalmente, caminhamos na contramão. Somos dependentes de matérias primas e tecnologias farmacêuticas importadas de outras nações. Por fim, além de mal utilizar nossos recursos vegetais, degradamos os ambientes nos quais boa parte das espécies se encontram inseridas (LOYOLA *et al.*, 2014; MARTINELLI; MESSINA; SANTOS FILHO, 2014; MARTINELLI; MORAES, 2013). A degradação do ambiente em dois dos nossos seis biomas é relatada pela comunidade científica (HRDINA; ROMPORTL, 2017; JENKINS; PIMM, 2006; MITTERMEIER *et al.*, 2011; MYERS *et al.*, 2000; REZENDE *et al.*, 2018). O Cerrado e a Mata Atlântica encontram-se entre as regiões do planeta entre as mais impactadas quanto à destruição, com aniquilação quase iminente de todas espécies vegetais e animais que ali residem (JENKINS; PIMM, 2006; MYERS *et al.*, 2000; REZENDE *et al.*, 2018). A falta de conhecimento científico a respeito das espécies vegetais é tida como um dos principais fatores que contribuem para sua extinção (MARTINELLI; MORAES, 2013), embora a diversidade é sempre associada a riqueza e a vantagens competitivas (CALIXTO, 2003; VILLAS BÔAS; GADELHA, 2007). Contudo, no Brasil ainda se faz muito pouco para transformar a biodiversidade em uma bioeconomia sustentável (EUROPEAN COMMISSION, 2015).

Frente a este cenário, almejamos neste trabalho darmos mais um passo para reconhecer a importância dos ambientes biodiversos, assegurando a eles ações de proteção. Num segundo

passo, ao adicionarmos conhecimento acerca do potencial farmacológico das espécies pouco estudadas vamos ao encontro da promoção de fatores que promovem a proteção, conforme relatado pelos trabalhos de Martinelli e cols (2014). Finalmente, associando a biodiversidade com conhecimento científico podemos produzir novas alternativas terapêuticas úteis aos tratamentos de saúde da população.

Neste sentido, entre as várias espécies nativas dos biomas impactados escolhemos trabalhar com alguns táxons da família Connaraceae. Do total de táxons de Connaraceae encontradas no Brasil, quase metade tem seu habitat nos biomas ameaçados do Cerrado e da Mata Atlântica, sendo as demais encontradas na Caatinga e na Floresta Amazônica (FORZZA *et al.*, 2010b; JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). Há relatos que algumas espécies de Connaraceae encontram-se ameaçadas de extinção (MARTINELLI *et al.*, 2018; MARTINELLI; MESSINA; SANTOS FILHO, 2014; MARTINELLI; MORAES, 2013). Entre os seis fitodomínios brasileiros, o Cerrado e a Mata Atlântica, são considerados áreas de *Hotspots* (MYERS *et al.*, 2000). *Hotspots* são áreas de elevada diversidade de espécies com alto grau de endemismo e marcadas pela elevada ação antrópica (MYERS *et al.*, 2000). Com isto, a conservação da biodiversidade destes espaços é tida como essencial e prioritária, visto que, eles concentram a maior parte da variedade genética de uma região ou país (JENKINS; PIMM, 2006). Neste sentido, mundialmente um primeiro momento foi estabelecido a existência de 25 áreas de *Hotspots* (MYERS *et al.*, 2000), embora, as revisões mais atuais indicam que no presente existem entre 35 e 36 pontos megadiversos no planeta (HRDINA; ROMPORTL, 2017; MITTERMEIER *et al.*, 2011).

Este projeto iniciou com uma ampla revisão do potencial farmacológico de espécies da família Connaraceae. Esta publicação abrange 39 espécies potencialmente úteis sobre a ótica da farmacologia e entre elas um terço podem ser encontradas no Brasil (PAIM *et al.*, 2020). Entre as principais atividades reportadas está o uso no controle do Diabetes (JIMÉNEZ *et al.*, 2001; LAIKOWSKI *et al.*, 2017; PANIAGUA ZAMBRANA *et al.*, 2017). Entre estes trabalhos que relatam atividades antidiabéticas estão estudos *in vitro* para *Connarus lambertii* (DC.) Sagot (JIMÉNEZ *et al.*, 2001) e *in vivo* de extratos obtidos de *Cnestis ferruginea* DC., *Rourea coccinea* (Schumach. & Thonn.) Benth., *Rourea minor* (Gaertn.) Alston, e de *Rourea cuspidata* Benth ex. Baker (ADISA *et al.*, 2010; AKINDELE *et al.*, 2014; DADA *et al.*, 2013; KULKARNI *et al.*, 2014; LAIKOWSKI *et al.*, 2017).

Entre estes estudos, um foi conduzido no Laboratório de Biotecnologia da Produtos Naturais e Sintéticos do Programa de Pós Graduação em Biotecnologia da Universidade de Caxias do Sul (LAIKOWSKI *et al.*, 2017), e resultou no depósito de patente frente aos promissores resultados que encontrou (LAIKOWSKI *et al.*, 2018). Assim, decidimos seguir com os possíveis efeitos de Connaraceae e seus metabólitos secundários sobre a ótica do controle das complicações do Diabetes. Trata-se de uma patologia, que apresenta alta prevalência no Brasil e no mundo e representa uma das principais causas de morte tanto em países desenvolvidos quanto naqueles em desenvolvimento (LADDHA; KULKARNI, 2019; MINISTÉRIO DA SAÚDE, 2020; SCHMIDT *et al.*, 2014). Desta forma, buscamos compreender os efeitos destes compostos ativos para além da redução da glicemia, atividade essa já relatada para diferentes taxa (ADISA *et al.*, 2010; DADA *et al.*, 2013; KULKARNI *et al.*, 2014; LAIKOWSKI *et al.*, 2017). Compostos químicos identificados por outros autores em Connaraceae suportam vislumbrar que essa família de plantas pode ser útil como um tratamento complementar aos hipoglicemiantes orais ou até mesmo a insulina.

Assim, este trabalho visou a evolução do conhecimento científico acerca de Connaraceae, com a adição de estudos sobre a composição química e estudos farmacológicos a partir das espécies *Connarus blanchetii* Planch. *Connarus nodosus* Baker, *Connarus regnellii* G.Schellenb, *Connarus suberosus* Planch., *Rourea glazioui* G. Schellenb e *Rourea induta* Planch além da *Rourea cuspidata* já previamente estudada por Laikoswki e cols (2017). Para isso, foi buscado o melhor método de extração, a qualificação via espectrofotometria, bem como a identificação dos principais compostos presentes nestes táxons utilizando cromatografia a liquido de alta eficiência (CLAE) com detector de espectrometria de massas de alta resolução (EMAR). Ainda, lançamos mão de plataformas de bioinformática como a *Global Natural Products Social* (GNPS) entre outras para confirmação das estruturas dos compostos presentes. Por fim, os extratos e frações foram testados *in vitro,* em relação à atividade antiglicante, antioxidante e inibidora de algumas enzimas associadas ao metabolismo de carboidratos (ADISA *et al.*, 2010; AKINDELE *et al.*, 2014; DADA *et al.*, 2013; KULKARNI *et al.*, 2014; LAIKOWSKI *et al.*, 2017), com intuito de indicarmos o potencial das espécies estudadas para uso como adjuvante no tratamento das complicações do diabetes. Em resumo, este trabalho busca promover o aumento do conhecimento sobre estas espécies, intencionando alternativas a minimizar a complicações a associadas ao Diabetes, e além disso, ampliar a possibilidade de proteção ambiental dos ambientes onde estas espécies habitam. Assim, imaginamos que a ciência seja uma ferramenta para a promoção de ações que visem à proteção da biodiversidade

brasileira, promovendo o uso racional e o aproveitamento destes recursos com a finalidade farmacológica.

2 REVISÃO DA LITERATURA

2.1 Connaraceae

A família Connaraceae distribui-se pelas regiões tropicais do planeta (FORERO, 2007) contempla 12 a 13 gêneros e aproximadamente 200 espécies (LEMMENS, 1989; LEMMENS; BRETELER; JONGKIND, 2004). Connaraceae possui relatos de distribuição botânica que incluem a América do Sul e Central (FORERO, 1981, 2007), a África (LEMMENS; BRETELER; JONGKIND, 2004) e a Ásia (LEENHOUTS, 1958). Connaraceae apresenta como principais características botânicas, figura 1, o desenvolvimento sob a forma de arbustos, lianas e árvores (LEENHOUTS, 1958), sendo portadora de folhas compostas, alternas, sem estípulas, imparipinadas e inflorescência do tipo paniculada ou racemosa (PASTORE *et al.*, 2017).

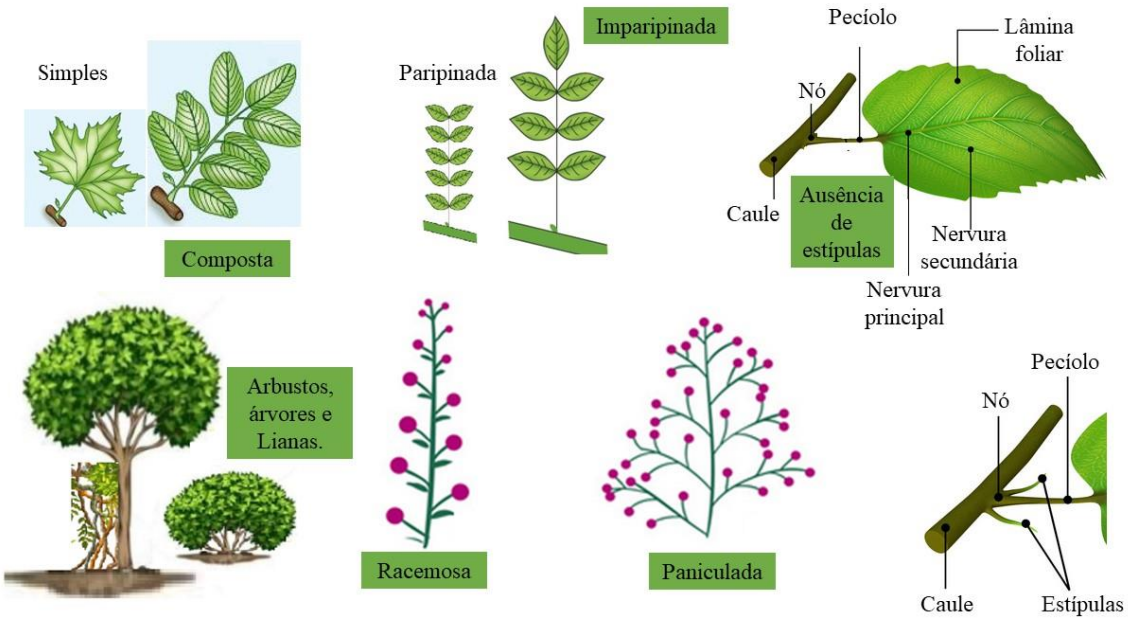

Figura 1: Características botânicas de Connaraceae.

Fonte: o autor.

No Brasil são relatadas a presença de 4 ou 5 gêneros de Connaraceae entre estes *Bernardinia*, *Cnestidium*, *Connarus*, *Pseudoconnarus* e *Rourea* (FORERO, 2002; FORZZA *et al.*, 2010a; MARTINELLI; MORAES, 2013; TOLEDO; SOUZA, 2019; TOLEDO; SOUZA; LUCAS, 2019). O número de gêneros ainda não é consensual pois a existência de *Bernardinia* ainda não é questão fechada entre os taxonomistas. Da mesma forma, o número total de táxons de Connaraceae presente nos biomas brasileiros não é consensual, variando entre 69 e 72 espécies (MARTINELLI; MORAES, 2013; PASTORE *et al.*, 2017). Entretanto, para fins dessa revisão uma estimativa foi feita com base no descrito sobre a biodiversidade vegetal brasileira

(FORZZA *et al.*, 2010a, 2010b). A partir do compilado de informações (tabela 1), foi estimada a presença de 61 espécies (JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). A aceitabilidade dos nomes científicos de cada espécie foi verificada no Site *The Plant List* (THE PLANT LIST, 2020). Adicionalmente, fez-se uma análise da distribuição territorial de Connaraceae no Brasil, a partir dos diferentes biomas, a fim de que fosse possível compreender melhor o grau de ameaça em que essa família se encontra.

O endemismo, fenômeno no qual uma espécie apresenta distribuição restrita em determinada região geográfica, é característica importante para a família Connaraceae (MARTINELLI; MORAES, 2013) e também foi avaliado. Frente a essa característica em uma análise a partir dos dados disponíveis na literatura, observa-se que o endemismo de Connaraceae no território Brasileiro, engloba 40 espécies do total de 61 descritas (FORZZA *et al.*, 2010b). A distribuição dos táxons frente aos gêneros de Connaraceae mostra que *Rourea* apresenta-se como o mais representativo contendo 34 espécies, seguido por *Connarus* com 23, *Pseudoconnarus* com 3 e *Cnestidium*, com apenas uma espécie descrita.

Tabela 1: Connaraceae no Brasil e dados sobre estudos farmacológicos.

Endemismo				
	Endêmicas		Não endêmicas	
n	40		21	
Distribuição das espécies por gênero				
	Cnestidium	*Connarus*	*Pseudoconnarus*	*Rourea*
n	1	23	3	34
Distribuição de espécies por bioma				
	Floresta Amazônica	Mata Atlântica	Caatinga	Cerrado
n	33	26	3	8
Distribuição de espécies por bioma exclusivas em áreas de *Hotspots* em relação ao total de espécies				
	Mata Atlântica		Cerrado	
n	23/26		5/8	
Total de espécies relatados em utilização etnofarmacológica				
	Cnestidium	*Connarus*	*Pseudoconnarus*	*Rourea*
n	0	3	2	2
Total de espécies relatados em estudos publicados				
	Cnestidium	*Connarus*	*Pseudoconnarus*	*Rourea*
n	0	4	1	3

Fonte: Dados coletados a partir das fontes (FORZZA *et al.*, 2010b; JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). *Hotspots**- Conforme classificação das áreas geográficas feitas pelos trabalhos de (MYERS *et al.*, 2000). O gênero *Bernardinia* por apresentar-se não consensual e apenas uma espécie não foi considerado para fins dessa revisão.

A distribuição de Connaraceae frente aos biomas brasileiros mostrou que a Floresta Amazônica contém o maior número, contando com 33 táxons, seguida pela Mata Atlântica com

26, Cerrado com 8 e Caatinga com 3 espécies. Algumas espécies possuem distribuição ampla e são encontradas em mais de um bioma brasileiro dentre elas: *C. suberosus* e *R. induta* por exemplo. Considerando somente as espécies de Connaraceae encontradas em áreas de *Hotspots*, são no total 34 espécies e entre essas 28 são endêmicas para o território brasileiro (FORZZA *et al.*, 2010b; JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). Segundo a literatura, a perda de habitat aliada à falta de conhecimento sobre as espécies, representam as maiores ameaças a essa e outras famílias de plantas (MARTINELLI; MORAES, 2013). No Brasil, as espécies *Rourea cnestidifolia* G.Schellenb., *Rourea pseudospadicea* G.Schellenb., (MARTINELLI; MORAES, 2013), *Connarus marginatus* Planch. (MARTINELLI *et al.*, 2018) e *Rourea chrysomalla* Glaz. ex G.Schellenb. (MARTINELLI; MESSINA; SANTOS FILHO, 2014) estão em perigo de extinção.

Sete espécies de Connaraceae brasileiras têm usos reportados na medicina tradicional sendo três do gênero *Connarus*, duas para *Rourea* e duas para *Pseudoconnarus*. Os usos populares de Connaraceae (tabela 2) reportados para o gênero *Connarus* referem-se as espécies *Connarus angustifolius* (Radlk.) G. Schellenb.; *Connarus favosus* Planch. e *C. suberosus*. Para *Pseudoconnarus* inclui a espécie *Pseudoconnarus. rhynchosioides* (Standl.) Prance e para *Rourea*, os táxons *R. cuspidata* e *R. induta*. Esses usos incluem desde doenças relacionadas ao sistema cardíaco (TAVEIRA *et al.*, 1988), ao metabolismo de carboidratos (LAIKOWSKI *et al.*, 2017), e à doenças consideradas emergentes e negligenciadas como é o caso da Doença de Chagas (KALEGARI *et al.*, 2014a).

Tabela 2: Espécies brasileiras com potencial etnofarmacológico e/ou descritas em estudos farmacológicos publicados. Continua

Espécies	Nomes populares	Etno-farmacologia	Compostos químicos identificados	Atividades farmacológicas relatadas	Referências
Connarus angustifolius (Radlk.) G. Schellenb.	Barbatimão do pará, marassacaca e muraçacaca.	Infecções do trato genito-urinário, estomatites, malária e dor de cabeça.	Ácido gálico, ferúlico, cafeico, catequina, rutina, quercitrina e resveratrol.	Atividade citotóxica contra adenocarcinoma humano de colón.	(COELHO-FERREIRA, 2009; DEFILIPPS; MAINA; CREPIN, 2004; PARACAMPO, 2011; PASTORE *et al.*, 2017; PIRES *et al.*, 2017; SUFFREDINI *et al.*, 2007; YAZBEK *et al.*, 2016)
Connarus detersus Planch.	Cabelo de Negro	Sem relatos de utilização.	Nenhum.	Inibição da atividade da enzima acetilcolinesterase.	(FARIAS *et al.*, 2013)
Connarus favosus Planch.	Veronica	Picadas de cobras.	Nenhum.	Atividade antioxidante, contra o veneno ofídico *Bothrops atrox venom* e efeito antibacteriano.	(GOMBEAU *et al.*, 2019; MOURA *et al.*, 2015; SILVA *et al.*, 2016)
Connarus suberosus Planch.	Tropeiro ou bico de papagaio, galinha-choca	Diarreia e problemas cardíacos.	Suberonona, rapanona, β-sitosterol, ácido oleico, geraniol, embelina, tectoquinona, emodina, plumbagina, 2-etilantraquinona, 1-cloroantraquinona; antrona; 2-(4-piridil)-1H-antra[1,2-di]imidazol-6,11-diona, krisolamina, antraquinona-2-ácido carboxílico, 4,5-dinitrocrisazina, 1-aminoantraquinona, 5,7,12,14-pentacenetetrona, antrarufina, sódio antraquinona-2-sulfonada, ácido bromaminico, 2-fenillantraquinona, alizarina, quinizarina, reinina, 1-amino-4-hidroxyantraquinona, 2-aminoantraquinona, 1,5-diaminoanthaquinona, dibromoaminoantraquinona, 1,4-bis(metilamino)antraquinona; 1,8-dicloroantraquinona; 1,5-dicloroantraquinona diacereina, bergenina, connarina, hemileiocarpian e leiocarpina.	Contra o protozoário causador da leishmaniose, atividade contra larvas do mosquito *Aedes aegypti* e atividade antifúngica.	(CHARNEAU *et al.*, 2015; COSTA *et al.*, 2014; MORAIS *et al.*, 2020; SILVA *et al.*, 2020; TAVEIRA *et al.*, 1988)

Pseudoconnarus macrophyllus (Poepp.) Radlk.	***	Não relatada no uso popular.	Nenhum.	Atividade citotóxica contra adenocarcinoma humano de colón.	(SUFFREDINI *et al.*, 2007)
Pseudoconnarus rhynchosioides (Standl.) Prance	Saracura	Exaustão, estimulante sexual e malária.	Nenhum		(PEDROLLO *et al.*, 2016)
Rourea cuspidata Benth ex. Baker	Cipo miraruina	Diabetes.	Zingerona, octadecanamida, catequina, quercetina-3-*O*-galactosídeo (hiperina), quercetina-3-*O*-α-arabinofuranosídeo (guaijaverina) e proantocianidina A2.	Atividade hipoglicemiante.	(LAIKOWSKI *et al.*, 2017)
Rourea doniana Baker	***	Não relatada no uso popular	Lupeol, lupenona, α-amirenona, β-amirenona, taraxerol 7,4'-dimetilkamferol, escopoletina, β-sitosterol, estigmasterol, daucosterol e estigmasterol glucosidio.	Não descrita.	(OLIVEIRA; LEMOS; CONSERVA, 2012; OLIVEIRA *et al.*, 2010)
Rourea induta Planch.	Hohocré, chapeudinha, pau-de-porco or campeira	Reumatismo e Doença de Chagas.	Ácido neoclorogênico, quercetina-3-*O*-galactosídeo (hiperina), quercetina-3-*O*-xilosídeo (reinoutrina), quercetina-3-*O*-α-arabinofuranosídeo (guaijaverina) quercetina e proantocianidina C1	Atividade hepatoproterora, antioxidante e antinoceptiva.	(KALEGARI *et al.*, 2011, 2014a, 2014b; RODRIGUES, 2007; YAZBEK *et al.*, 2016)

Assim, a revisão quanto ao número de metabólitos secundários identificados em táxons brasileiros de Connaraceae revelou que *C. angustifolius* possui 7 compostos conhecidos (PIRES *et al.*, 2017) para *C. suberosus* 37 constituintes (COSTA *et al.*, 2014; MORAIS *et al.*, 2020; SILVA *et al.*, 2020), *R. cuspidata possui* 6 compostos (LAIKOWSKI *et al.*, 2017) e finalmente os trabalhos com *R. induta* descreveram outros 6 metabólitos (KALEGARI *et al.*, 2011, 2014a). No contexto das atividades farmacológicas relatadas para Connaraceae, podem ser encontradas publicações acerca de *C. angustifolius* (PIRES *et al.*, 2017), *C. detersus* (FARIAS *et al.*, 2013), *C. favosus* (GOMBEAU *et al.*, 2019; SILVA *et al.*, 2016), *C. suberosus* (COSTA *et al.*, 2014; MORAIS *et al.*, 2020; TAVEIRA *et al.*, 1988), *P. macrophyllus* (SUFFREDINI *et al.*, 2007), *R. cuspidata* (LAIKOWSKI *et al.*, 2017) e *R. induta* (KALEGARI *et al.*, 2011, 2014a, 2014b). Através desses dados estimou-se que em torno de apenas 10% dos táxons brasileiros foram estudados até o presente, reafirmando o amplo caminho que ainda precisa ser trilhado a fim de que se possa compreender melhor o potencial químico e biotecnológico dessa família de plantas.

2.2 Espécies de Connaraceae estudadas

Na sequência, estão descritas, resumidamente, as características botânicas e eventualmente outros dados relacionados à utilização etnofarmacológica e/ou estudos anteriormente conduzidos para as sete espécies estudadas neste trabalho. Essa pesquisa contempla 4 táxons pertencentes ao gênero *Connarus* e 3 táxons agregados ao gênero *Rourea*.

Connarus blanchetii

Connarus blanchetii, figura 2, é uma espécie arbustiva, contendo folhas compostas com 1 a 3 lâminas foliares cada, é endêmica do Brasil, de hábito terrícola podendo ser encontrada em matas do tipo restinga. Sua distribuição está descrita para o bioma Mata Atlântica tendo sido relatada nos estados da Bahia, Alagoas e Sergipe (JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). A revisão da literatura apontou que até o presente nenhum trabalho científico com vistas a elucidar a composição química da espécie foi conduzido. Adicionalmente, não foram encontrados relatos de utilização etnobotânica para essa espécie vegetal.

Figura 2: Ramo de *C. blanchetii* em processo de frutificação.
*Imagem gentilmente cedida por Cássio Augusto Patrocínio Toledo

Connarus nodosus

Connarus nodosus, figuras 3 e 4, é uma arvoreta (árvore de pequeno porte), contendo folhas 3 a 5 folioladas, endêmica do Brasil, de hábito terrícola podendo ser encontrada em matas do tipo restinga (JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). É conhecida pelo nome popular de mata-cachorro (CURTI, 2015), embora não haja relatos de toxicidade descritos em publicações para essa espécie. Sua distribuição está descrita para o bioma Mata Atlântica tendo sido relatada nos estados do Espirito Santo e do Rio de Janeiro (JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). A revisão da literatura apontou que até o presente nenhum trabalho científico abordou a composição química dessa espécie vegetal.

Figura 3: *Connarus nodosus* detalhe para o porte arvoreta.
*Imagem gentilmente cedida por Cássio Augusto Patrocínio Toledo

Figura 4: Ramo de *C. nodosus* em processo de frutificação.
*Imagem gentilmente cedida por Cássio Augusto Patrocínio Toledo

Connarus regnellii

Connarus regnellii, figuras 5 e 6, é uma espécie arbustiva, popularmente conhecida pelo nome de Camboatá da Serra, (ARVORES DO BRASIL, 2020) contendo folhas compostas com 3 a 5 lâminas foliares cada, é endêmica do Brasil, de hábito terrícola podendo ser encontrada na floresta estacional semidecidual. Sua distribuição está descrita para o bioma Mata Atlântica tendo sido relatada nos estados do Minas Gerais, São Paulo e Rio de Janeiro (JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). A revisão da literatura apontou que até o presente nenhum trabalho científico abordou a composição química dessa espécie vegetal. Entretanto, há relatos dessa espécie como árvore usada para fins de ornamentação com disponibilidade de sementes e mudas as quais podem ser encontradas comercialmente.

Figura 5: Ramo de *C. regnellii* em processo de frutificação.
Imagem gentilmente cedida por Cássio Augusto Patrocínio Toledo

Figura 6: Frutos de *C. regnellii.*
Imagem gentilmente cedida por Cássio Augusto Patrocínio Toledo

Connarus suberosus

Connarus suberosus, figuras 7 e 8, é uma árvore, popularmente conhecida pelo nome de Cabelo-de-negro (ARVORES DO BRASIL, 2020), Tropeiro ou Bico de Papagaio (TAVEIRA *et al.*, 1988) de casca muito suberosa característica que lhe confere o nome (COSTA *et al.*, 2014), contendo folhas compostas de 5 ou mais lâminas foliares cada, não é endêmica do Brasil, de hábito terrícola podendo ser encontrada no Cerrado Brasileiro. Sua distribuição é ampla e descrita em quase todos os estados cobertos por este bioma incluindo o Pará, Tocantins, Bahia, Maranhão, Piauí, Distrito Federal, Goiás, Mato Grosso do Sul, Mato Grosso, Minas Gerais e São Paulo (JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). Essa espécie vegetal possui relatos de utilização etnofarmacológica (COSTA *et al.*, 2014; TAVEIRA *et al.*, 1988), destacando-se os usos referentes à melhora da função cardíaca (TAVEIRA *et al.*, 1988) e no tratamento da diarreia (COSTA *et al.*, 2014). O

efeito promissor dessa espécie vegetal foi demonstrado frente ao protozoário *Leishmania* (*Leishmania*) *amazonenses* e frente à diversos fungos dos gêneros *Candida* e *Trychophytum* (COSTA *et al.,* 2014). Trinta e sete compostos químicos foram identificados no extratos dessa planta até o presente, dentre eles rapanona e suberona (COSTA *et al.,* 2014; MORAIS *et al.,* 2020). Entretanto, essa espécie vegetal não possui reserva de patente frente a estes ou quaisquer outros efeitos biológicos a ela atribuídos.

Figura 7: Caule suberoso de *C. suberosus*. Imagem gentilmente cedida por Joicelene Regina Lima da Paz.

Figura 8: Ramo de *C. suberosus*. Fonte: Arquivo Flickr.com

Rourea cuspidata

Rourea cuspidata, figuras 9 e 10, é uma espécie trepadeira (liana), contendo folhas compostas de 3 a 5 lâminas foliares cada, não é endêmica do Brasil, de hábito terrícola podendo ser encontrada na Floresta Ciliar ou de Galeria, Floresta de Igapó, Floresta de Várzea e Floresta de Terra Firme. Sua distribuição está descrita para o bioma Floresta Amazônica tendo sido relatada nos estados do Acre, Amazonas, Pará, Rondônia e Roraima (JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). Essa espécie vegetal tem relatos de utilização etnofarmacológica no controle do diabetes, sendo essa utilização confirmada em ensaios *in vivo* em trabalho prévio do nosso grupo de pesquisa (LAIKOWSKI *et al.,* 2017). Adicionalmente, essa espécie vegetal tem reserva de patente em invenção, que descreve uma composição farmacêutica compreendendo extrato padronizado de *Rourea cuspidata* e seus derivados compreendendo o uso desta composição para o tratamento da Diabetes *Mellitus* (DM) (LAIKOWSKI *et al.,* 2018).

Figura 9: Caule do tipo de *R. cuspidata*.
Imagem gentilmente cedida por Manuela M. Laikowski.

Figura 10: Ramo de *R. cuspidata.*
Imagem gentilmente cedida por Manuela M. Laikowski.

Rourea glazioui

Rourea glazioui, figura 11, é uma espécie trepadeira (liana), contendo folhas compostas com mais de 15 lâminas foliares cada, é endêmica do Brasil, de hábito terrícola podendo ser encontrada na Floresta Ombrófila (Floresta Pluvial). Sua distribuição está descrita para o bioma Mata Atlântica, tendo sido descrita nos estados da Bahia, Espírito Santo e Rio de Janeiro (JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). Até o presente nenhum trabalho científico abordou a composição química dessa espécie vegetal e também não possui reserva de patente.

Figura 11: Ramo de *R. glazioui* em processo de frutificação.
Imagem gentilmente cedida por Cássio Augusto Patrocínio Toledo.

Rourea induta

Rourea induta, figura 12, é uma arvoreta, contendo folhas compostas de 5 a 9 lâminas foliares cada, não é endêmica do Brasil, de hábito terrícola podendo ser encontrada no Cerrado Brasileiro. Sua distribuição é ampla e descrita em quase todos os estados cobertos por este bioma incluindo o Pará, Rondônia, Tocantins, Bahia, Ceará, Maranhão, Pernambuco, Piauí,

Distrito Federal, Goiás, Mato Grosso do Sul, Mato Grosso, Minas Gerais e São Paulo (JARDIM BOTÂNICO DO RIO DE JANEIRO, 2020). Essa espécie vegetal possui relatos de utilização etnofarmacológica, como no tratamento da artrite e Doença de Chagas, como antinoceptiva, hepatoprotetora e antioxidante (KALEGARI *et al.*, 2014b, 2014a). Adicionalmente, essa espécie vegetal tem reserva de patente em invenção que descreve as propriedades medicinais, nutricionais, farmacêuticas, cosméticas, microbiológicas, alelopáticas, imunológicas, anti-inflamatórias, antiparasitárias, contra o *Trypanosoma cruzi*, e ação antioxidante no campo humano, veterinário e ambiental da espécie (DALLARMI *et al.*, 2012).

Figura 12: Ramo de *R. induta* em processo de frutificação.
Fonte: Arquivo Flickr.com

2.3 Ferramentas de bioinformática para estudo dos dados gerados pela cromatografia líquida acoplada a espectrometria de massas CL/EM.

A espectrometria de massas (EM) é uma poderosa ferramenta para análise da composição química em misturas complexas a qual possibilitou avanços em diversas áreas da ciência, incluindo a metabolômica (LANÇAS, 2019). Entre as várias aplicações da EM o estudo do metabolismo vegetal secundário tem sido empregado por diversos autores (BEN SAID *et al.*, 2017; KIND; FIEHN, 2017; KITE; VEITCH, 2011; SANTOS *et al.*, 2020). Entre as vantagens da EM frente a outras técnicas está a necessidade de pequenas quantidades de amostras as quais podem fornecer muita informações (BOUSLIMANI *et al.*, 2014) e também uma alta sensibilidade analítica permitindo a identificação de substâncias em baixíssimas concentrações (YANG *et al.*, 2013). Assim, temos uma técnica amplamente aceita e que vem

sendo utilizada nos mais diversos campos de pesquisa, incluindo as ciências biológicas, farmacêuticas, identificação de metabólitos e a análise de peptídeos (ALLEN; MCWHINNEY, 2019). Trata-se de um equipamento onde os compostos são inseridos (via cromatografia, eletroforese, ou infusão direta), posteriormente os compostos são ionizados numa fonte (carregados positiva ou negativamente), os quais passam na sequência por um processo de seleção das massas e por fim os mesmos são analisados e detectados, figura 13.

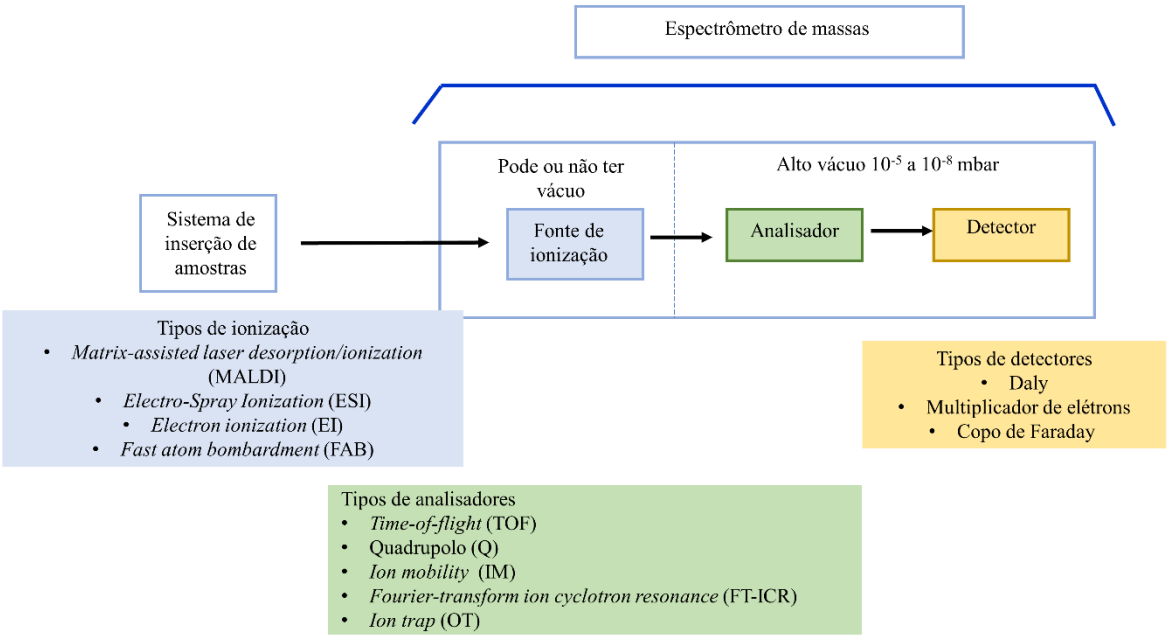

Figura 13: Modelo esquemático da espectrometria de massas.
Fonte: o autor.

Boa parte da evolução da EM tem relação com o desenvolvimento de ionização por *electrospray* (ESI) (URBAN, 2016). A ESI permite analisar moléculas de alto peso molecular, como proteínas e polímeros de ácidos nucleicos (GROSS, 2020), bem como, moléculas de baixo peso molecular, incluindo a maioria dos metabólitos secundários vegetais. Aparelhos do tipo MS Q-TOF-MS combinam os benefícios de dois analisadores de massas diferentes, assegurando alta eficiência de fragmentação de compostos aliada a rápida velocidade de análise a alta capacidade de resolução (ALLEN; MCWHINNEY, 2019).

Atualmente, estão disponíveis vários tipos de espectrômetros de massas com os mais diversos analisadores de massa como por exemplo: por tempo de voo do inglês *time-of-flight* (TOF), ressonância de íon ciclotron por transformada de Fourier do inglês *Fourier-transform ion cyclotron resonance* (FT-ICR) e analisadores de massa do tipo orbitrap do inglês *ion trap*

(OT) (ALLEN; MCWHINNEY, 2019). Para alcançar resoluções superiores a 15.000 FWHM, estes analisadores são colocados em sequência (do inglês *Tandem*), sendo assim, temos equipamentos com analisadores de massas com: Quadrupolo – Tempo de voo (Q-TOF), ou Quadrupolo – Trap (Q-trap), entre outros. Esses aparelhos utilizam técnicas que diferem entre si. Nos aparelhos do tipo TOF usa-se a técnica tempo dispersão mediada por um feixe de íons pulsados e esses são separados em razão ao seu tempo de voo, para FT-ICR os íons são presos num campo magnético e separação por se dá através da frequência cicloton e a detecção é do tipo corrente de imagem pela transformação de Fourrier de um sinal transitório. Finalmente, o tipo OT trabalha com a oscilação axial em campo elétrico não homogêneo e a deteção de frequência acontece pela transformação de Fourrier de um sinal transitório (GROSS, 2020). Em comum, todos os espectrômetros de massas combinam um sistema de injeção, ionização, analisador de massas e detecção, embora os maiores avanços nesta técnica analítica estão relacionados ao sistema de ionização que cada aparelho possui.

Todos os espectrômetros de massas geram uma infinidade de dados, sendo que a análise destas informações demandam muito tempo, sem aplicação de ferramentas computacionais adequadas (AMARAL, 2018). Por vezes, o estudo dos íons detectados num espectro de massas pode ser feito manualmente a partir de bibliotecas espectrais, as quais contém a razão massa carga para as substâncias conhecidas. Até pouco tempo, a identificação de moléculas em misturas complexas estava entre as tarefas mais desafiadoras enfrentada por pesquisadores na área de produtos naturais, pois haviam poucas ou nenhuma ferramentas computacionais capazes de compilar a infinidade de espectros de massas gerados pelos equipamentos (BOUSLIMANI *et al.*, 2014). Com o advento da bioinformática diferentes plataformas de análise computacional aplicáveis na interpretação dos dados da EM vêm sendo disponibilizadas (ALLEN *et al.*, 2014; DJOUMBOU-FEUNANG *et al.*, 2019; PATINY; BOREL, 2013; WANG *et al.*, 2016b). Essas ferramentas facilitaram o trabalho dos pesquisadores na desreplicação e na identificação de compostos com potencial farmacológico (LIMA *et al.*, 2021; SANTOS *et al.*, 2021). Assim, alternativamente, ao trabalho manual e laborioso, a interpretação dos espectros de massas pode agora ser conduzido em plataformas online que permitem analisar grandes quantidades de dados em curtos intervalos de tempo (WANG *et al.*, 2016b).

A desreplicação é um processo de identificação de moléculas onde os compostos já tiveram suas estruturas químicas elucidadas, sendo associado à estas um espectro de fragmentação, além de informações de massa exata e razão isotópica. Estas informações são depositadas numa base de dados permitindo a outros pesquisadores usá-las para caracterização

estrutural. A desreplicação é um ponto crítico para a descoberta dos constituintes associados ao metabolismo secundário vegetal (BOUSLIMANI *et al.*, 2014).

Neste sentido, a plataforma *Global Natural Products Social GNPS* (https://gnps.ucsd.edu) – molecular networking surgiu em meados de 2016 permitindo aos pesquisadores o compartilhamento e a curadoria de dados de espectrometria de massas associadas aos produtos naturais (WANG *et al.*, 2016a). Nesta é possível acessar algumas ferramentas uteis para caracterização de compostos em misturas complexas como extratos de plantas. (WANG *et al.*, 2016b). Entre elas, estão as redes moleculares do inglês (*molecular networking*) a quais permitem a construção de representações visuais do espaço químico que podem ser construídas a partir de experimentos de EM. Nas redes moleculares, as correlações espectrais detectam e agrupam moléculas quimicamente relacionadas, assumindo que essas moléculas se fragmentam de maneira semelhante aos seus padrões de espectrometria de massas em tandem (MS/MS) (WANG *et al.*, 2016b). A figura 14 ilustra uma rede molecular construída na plataforma GNPS e editada na programa Cytoscape®. Esta apresenta as desreplicações dos flavonóis camferol, quercetina e miricetina. Para a construção da rede molecular a tolerância de massa para o íon precursor e seus fragmentos deve estar ajustada para as condições de análise compatíveis com a capacidade de resolução do espectrômetro de massas utilizado no experimento. É necessário considerar, ainda, entre os parâmetros de análise qual o número de íons fragmentos será usado para a comparação entre os espectros de massas para os diferentes metabólitos. Assim o número de fragmentos compartilhados deve estar de acordo com a tendência da molécula em fragmentar-se ou não. Neste caso, estão representados compostos que compartilham pelo menos 6 íons fragmentos.

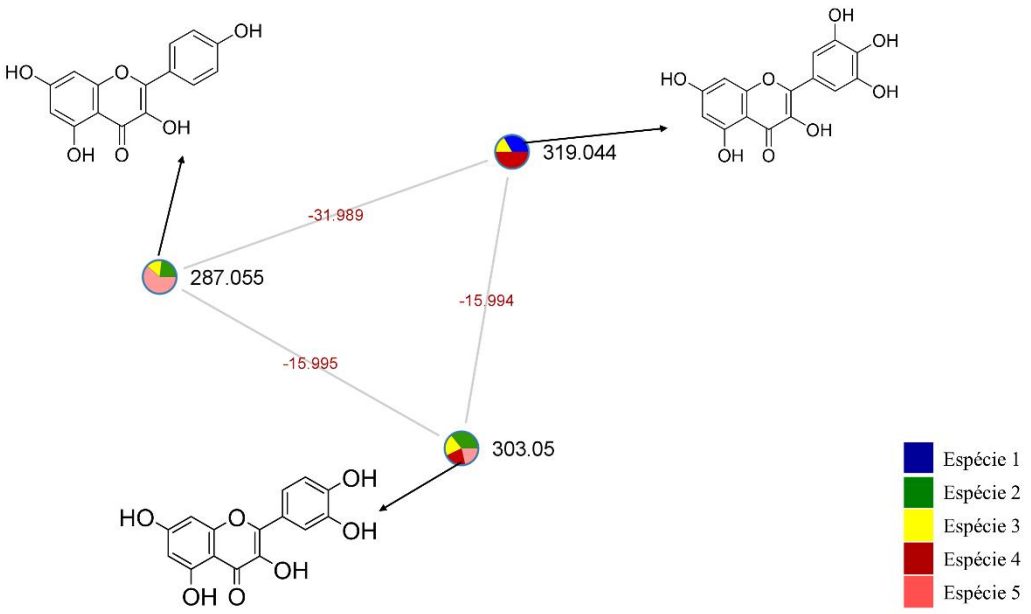

Figura 14: Exemplo de rede molecular obtida na Plataforma GNPS.
Fonte: o autor.

Este *cluster* com três metabólitos: canferol, quercetina e miricetina, foi construído através das relações matemáticas de pontuação de cosseno, indicadas pelo usuário e calculadas pela ferramenta, a qual permitiu estabelecer o agrupamento molecular. Assim, cada nodo representa um composto diferente, as cores indicam em quais amostras o metabólito foi detectado. Os conjuntos de moléculas dentro da rede molecular e seus diferentes clusters podem conter compostos desconhecidos, embora, se pertencentes ao mesmo agrupamento são quimicamente relacionados (WANG *et al.*, 2016b), ou seja, um *cluster* poder conter compostos desreplicados e também compostos não-desreplicados.

Além da GNPS outras ferramentas de bioinformática podem ser úteis na busca de identificação dos compostos químicos a partir da EM. A integração destas é tida como um ponto de inflexão metodológico para a descoberta de produtos naturais e também para a triagem metabolômica a qual pode prover as condições para aproveitamento do poder analítico dos espectrômetros de massas (QUINN *et al.*, 2017). Considerando que as análises feitas por redes moleculares podem conter *clusters* com compostos desreplicados e não-desreplicados, alternativas complementares de análise, providas por outras ferramentas de bioinformática podem ser utilizadas para a complementação da tentativa de identificação de compostos químicos. Entre essas ferramentas a modelagem de fragmentação competitiva e calculadora de massas isotópicas podem ser consideradas de grande utilidade. A modelagem de fragmentação

competitiva é uma ferramenta que produz um modelo teórico para sequencia de fragmentação para um composto de interesse permitindo ao analista comparar o modelo teórico ao experimental, bem como, propor a estrutura química dos fragmentos relacionados (ALLEN *et al.*, 2014; DJOUMBOU-FEUNANG *et al.*, 2019). As calculadoras de massas isotópicas (PATINY; BOREL, 2013) permitem a busca pelas possíveis fórmulas moleculares que podem estar relacionadas à determinado íon fragmento, ou até mesmo definir a formula molecular que produz a diferença entre as massas de dois metabólitos ligados por uma ponte numa rede molecular.

Na figura 15 a seguir temos o exemplo genérico do composto 1 (em verde) o qual teve a desreplicação alcançada via GNPS – *molecular networking* e foi identificado como quercetina-3-*O*-ramnosídeo ($C_{21}H_{20}O_{11}$) e composto 2 (em vermelho) cuja desreplicação não pode ser obtida.

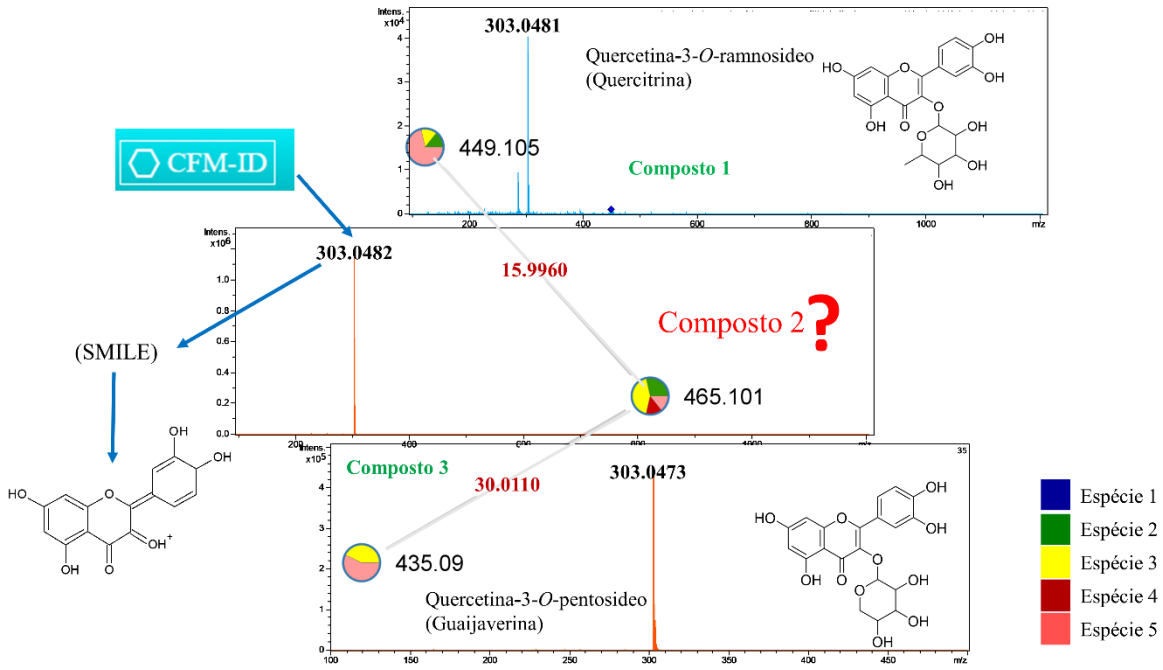

Figura 15: Modelo esquemático da complementação do processo de tentativa de identificação através da plataforma CFM-ID – predição espectral.
Fonte: o autor.

Os espectros de massas mostram que ambos os compostos geram o íon fragmento *m/z* 303.0481 como o mais intenso. Na análise via predição espectral, através do modelo teórico, é possível verificar que quercetina-3-*O*-ramnosídeo $[M + H]^+$ 449.1051 *m/z* probabilisticamente produzirá o íon fragmento 303.0481 como o mais intenso e que esse íon refere-se a um fragmento com *Simplified Molecular Input Line Entry Specification* (SMILE)

(O=C1C(=[OH+])C(=C2C=CC(O)C(O)=C2)OC2=CC(O)=CC(O)=C12) cuja fórmula química é $C_{15}H_{11}O_7^+$ compatível com a forma ionizada de quercetina. Portanto, considerando que os compostos 1 e 2 produzem o mesmo íon fragmento estes serão heterosídeos de quercetina.

Ainda, a figura 16, demonstra a busca de massas isotópicas na plataforma MF FINDER ChemCalc (PATINY; BOREL, 2013). A partir das diferenças entre as massas dos compostos 1 e 2 é possível inferir que há 15.996 Daltons (Da) a qual é compátivel com um átomo de oxigênio. Por outro lado, a diferença entre as massas dos compostos 2 e 3, que é igual a 30.011 Da, a qual refere-se a formula molecular CH_2O.

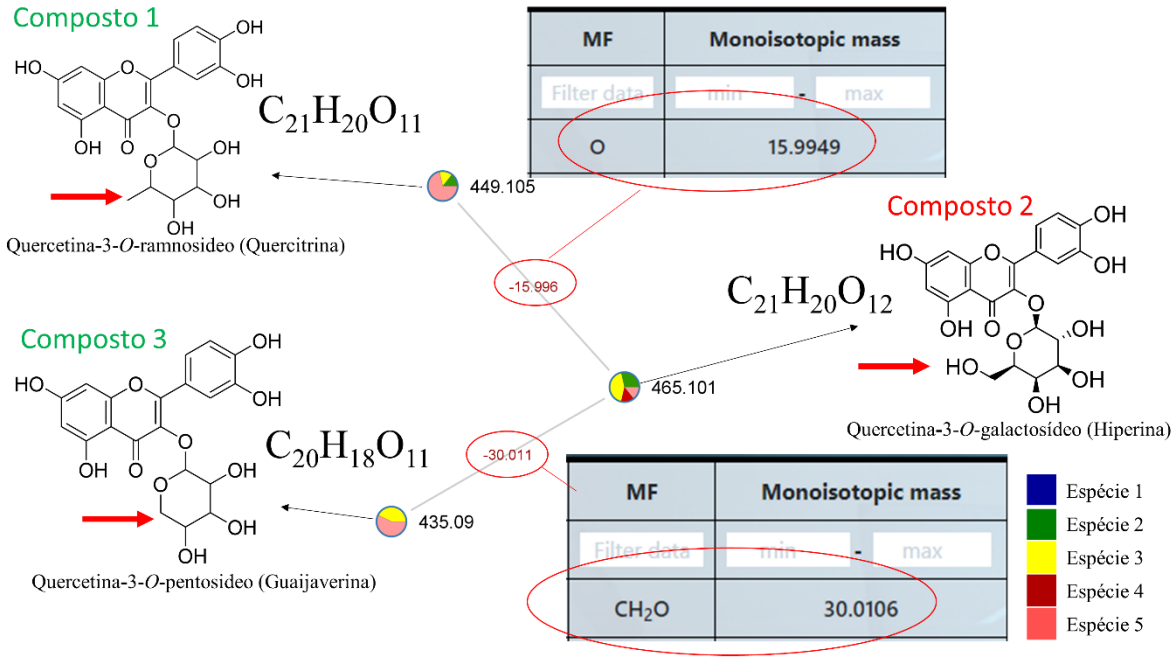

Figura 16: Modelo esquemático da complementação do processo de tentativa de identificação através da plataforma MF-Finder – ChemCalc.
Fonte: o autor.

Portanto, os compostos 1 e 2 diferem entre si pela presença de um átomo de oxigênio sendo possível estabelecer que se a formúla química do metabólito 1 é ($C_{21}H_{20}O_{11}$) a do composto 2 será ($C_{21}H_{20}O_{12}$) compatível com o quercetina-3-*O*-galactosídeo. A mesma formula para o composto 2 poderia ser obtida pela diferença observada em relação ao composto 3 ($C_{20}H_{18}O_{11}$). Desse modo, associando as informações produzidas pela rede molecular com predição espectral e busca de fórmulas moleculares a partir das massas isotópicas é possível propor a identidade de bom número de metabólitos em determinado *cluster*. Assim, para uma s

correta caracterizacão quimica, é necessario a combinacao de diferentes ferramentas de bioinfomática.

2.4 Diabetes: suas complicações e o potencial dos metabólitos secundários de Connaraceae como adjuvantes no tratamento farmacológico

O Diabetes *Mellitus* faz parte de um grupo de doenças associadas a desordens do metabolismo sendo caracterizada por elevados níveis de glicose no sangue, sendo causada pela insuficiência na produção ou ao efeito da insulina em seus tecido alvo (LI *et al.*, 2016; MARITIM; SANDERS; WATKINS, 2003). O DM uma doença que afeta 13 milhões de Brasileiros, cerca de 7 % do total da população (MINISTÉRIO DA SAÚDE, 2020). Segundo a Organização Mundial da Saúde (OMS), o diabetes junto com as doenças cardiovasculares, cânceres e doenças respiratórias crônicas formam o grupo das doenças não transmissíveis as quais respondem pelas principais causas de mortes no mundo (OMS, 2020).

Segundo a Associação Americana de Diabete (AMERICAN DIABETES ASSOCIATION, 2021) essa patologia pode ser classificada em três tipos principais:

1. Diabetes tipo I: decorrente da destruição mediada por via imune das células beta pancreáticas levando à deficiência de insulina;
2. Diabetes tipo II: ocorre por um defeito na secreção de insulina e/ou pela resistência à ela;
3. Diabetes *Mellitus* gestacional: relacionada a intolerância à glicose no início ou primeiro reconhecimento da gravidez.

Embora sejam doenças patologicamente distintas as diferentes classificações do Diabetes apresentam em comum a existência de hiperglicemia (FIORELLO *et al.*, 2020). Neste contexto o tratamento adequado dessa patologia precisa ser introduzido precocemente de modo a evitar as complicações associadas (DRZEWOSKI; KASZNICKI; TROJANOWSKI, 2009).

O tratamento do diabetes pode ser feito com hipoglicemiantes orais ou insulinas, sendo que muitos destes podem ser acessados pelos usuários do Sistema Único de Saúde (SUS). No Componente Básico da Assistência Farmacêutica (CBAF) ou através do Programa Aqui tem Farmácia Popular (BRASIL, 2021b, 2021a) os brasileiros têm acesso a vários tipos de medicamentos para o Diabetes de forma gratuita. Embora o acesso aos medicamentos esteja assegurado verifica-se mesmo entre os pacientes tratados existe alta taxas de internações hospitalares e complicações relacionadas a cetoacidose diabética, complicações circulatórias periféricas e coma (ARTILHEIRO *et al.*, 2014). Portanto, mesmo diante da disponibilidade de

medicamentos pelo SUS e pelo Programa Aqui tem Farmácia Popular de forma gratuita muitos pacientes ainda apresentam as complicações associadas a doença.

A hiperglicemia crônica associada a um inadequado do controle glicêmico, ou ao início tardio do tratamento do diabetes está implicada a uma condição conhecida como memória metabólica (CERIELLO; IHNAT; THORPE, 2009). A memória metabólica no diabetes refere-se a alterações genômicas que são induzidas pela cronificação da hiperglicemia em pacientes que não fazem um controle precoce e adequado nos níveis de glicose circulantes (CERIELLO; IHNAT; THORPE, 2009). Essa hiperglicemia crônica cria um ambiente no qual doenças relacionadas ao diabetes podem levar ao desenvolvimento de uma sequência de eventos denominadas de ciclo vicioso da memória metabólica (DRZEWOSKI; KASZNICKI; TROJANOWSKI, 2009), conforme ilustrado pela figura 17 a seguir:

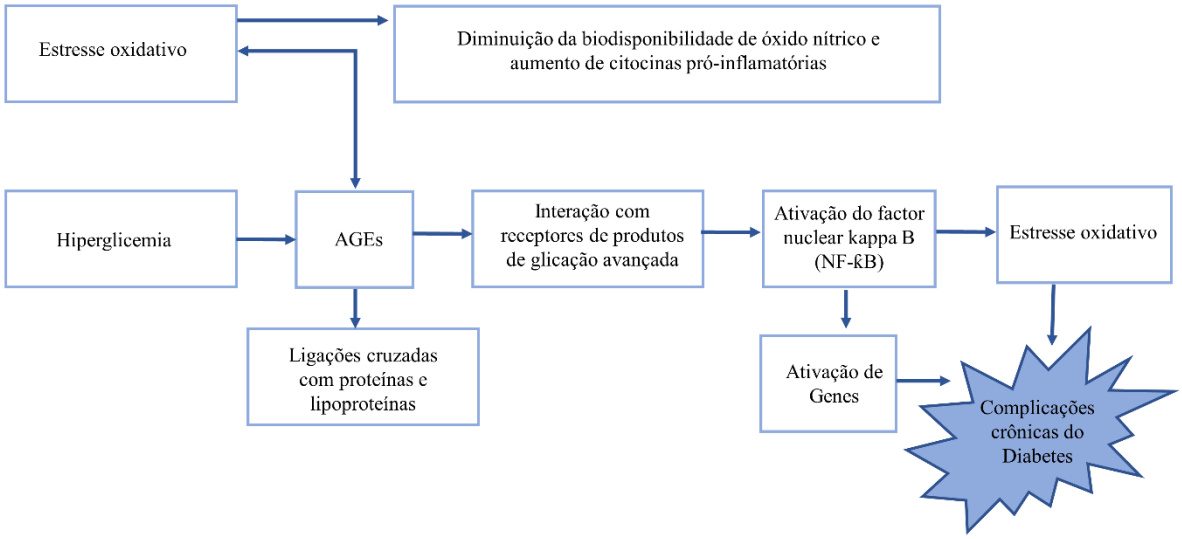

Figura 17: O ciclo vicioso da memória metabólica
Fonte: (DRZEWOSKI; KASZNICKI; TROJANOWSKI, 2009) adaptado.

As complicações do diabetes tabela 3 podem ser divididas em agudas e crônicas quanto ao tempo e em macro e microvasculares quanto ao calibre do sistema circulatório envolvido (ASMAT; ABAD; ISMAIL, 2016).

Tabela 3: Complicações do diabetes.

Complicações agudas	**Complicações crônicas**
Infecções	Cegueira e retinopatia
Cetoacidose	Neuropatia
Hiperglicemia,	Aterosclerose
Sede excessiva, poliúria, fatiga e visão borrada	Doença vascular periférica
	Infecções e amputações
	Doenças cerebrovasculares
Macrovasculares	**Microvasculares**
Derrames	Retinopatia e catarata
Doença cardíaca e hipertensão	Doença renal
Doença vascular periférica	Neuropatia
Problemas nos pés	Problemas nos pés (circulação)

Fonte: (ASMAT; ABAD; ISMAIL, 2016), adaptado.

A hiperglicemia diabética resulta em um aumento na produção de radicais livres por um mecanismo que envolve a oxidação da glicose seguida pela glicação de proteínas (MARITIM; SANDERS; WATKINS, 2003). O envolvimento dos processos mitocondriais na exacerbação do estresse oxidativo em resposta a hiperglicemia está implicada com as complicações do Diabetes (CERIELLO; IHNAT; THORPE, 2009). Na figura 18 a seguir apresenta um modelo esquemático dessas alterações.

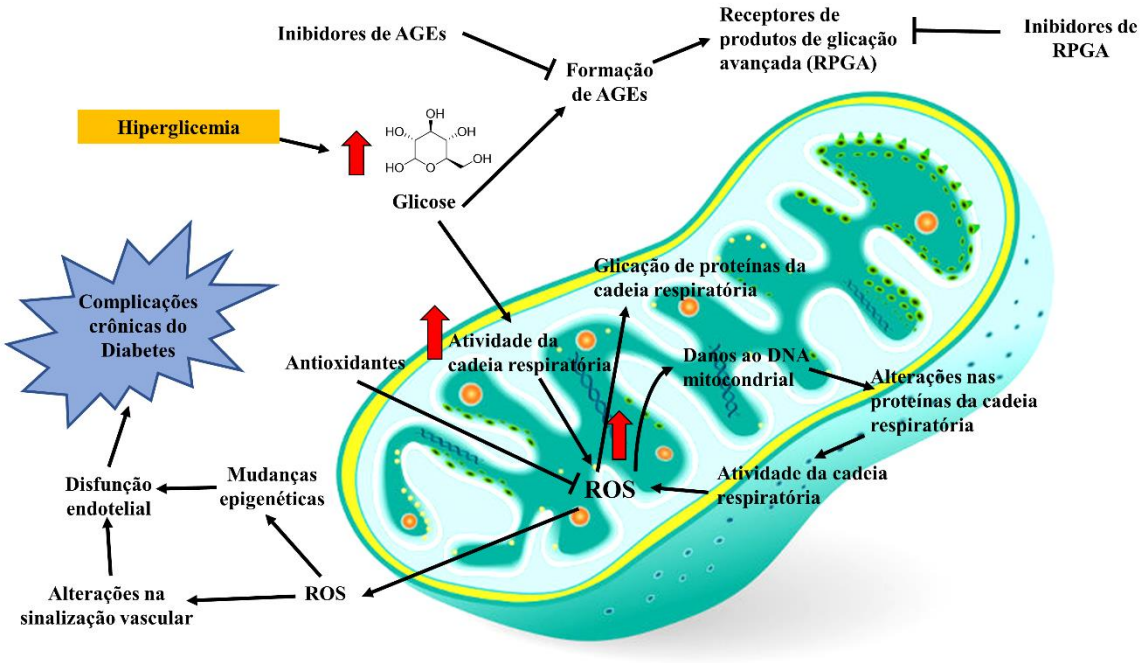

Figura 18: O envolvimento dos processos mitocondriais na hiperglicemia
Fonte: *(CERIELLO; IHNAT; THORPE, 2009)* adaptado.

Portanto a hiperglicemia é capaz de promover alterações na atividade da cadeia respiratória, bem como, é determinante para a formação dos produtos de glicação avançada. As alterações na geração de espécies reativas de oxigênio em inglês *reactive oxygen species* (ROS) em nível mitocondrial levam a danos ao DNA e subsequente alterações nas proteínas envolvidas com a respiração (CERIELLO; IHNAT; THORPE, 2009). Adicional e externamente a mitocôndria as espécies reativas de oxigênio promovem alterações epigenéticas e na sinalização vascular as quais finalmente estão implicadas com a disfunção endotelial e as complicações associadas ao diabetes (CERIELLO; IHNAT; THORPE, 2009). Em células endoteliais a exposição a altos níveis de glicose promove alterações na constrição as quais estão relacionadas com o fator de risco a microtromboses (FIORELLO *et al.*, 2020), portanto pacientes diabéticos com controle glicêmico inadequando podem ser considerados potencialmente mais propensos a desenvolver eventos trombóticos.

A modificação não enzimática de proteínas na hiperglicemia, entre elas a glicação, é o outro evento envolvido com as complicações diabéticas (CHETYRKIN *et al.*, 2011). O processo de glicação segue uma sequência de reações que podem ser reversíveis ou não (MARITIM; SANDERS; WATKINS, 2003). Na primeira etapa, ocorre a formação de uma base Schiff instável, processo que pode demorar cerca de 4 dias, sendo esse processo reversível. Na etapa posterior que ocorre em até 28 dias, há a reorganização da base Schiff que dá origem a um produto Amadori estacionário, processo que também pode ser reversível, embora se não

houver a remoção ou renovação desse intermediário o processo dará origem aos produtos finais de glicação do inglês *Advanced Glycation End Product* (AGEs) sendo esse último de natureza irreversível. É importante destacar que o processo de glicação proteica ocorre de modo espontâneo e não dependente de enzimas sendo no diabetes ocasionado pela reação da glicose com grupos amina terminais ou com resíduos do aminoácido lisina (RABBANI; THORNALLEY, 2021). A reação tem natureza covalente e ocorre entre o grupo carbonil do açúcar redutor e os aminoácidos da proteína (LADDHA; KULKARNI, 2019). Os AGEs formados são os principais mediadores patogênicos das complicações diabéticas, incluindo a retinopatia, a nefropatia e a neuropatia diabética (TORRES *et al.*, 2018).

Portanto a busca por compostos químicos que possam inibir o processo de glicação proteica pode trazer grandes benefícios à saúde dos pacientes diabéticos. Algumas espécies vegetais, de outras famílias botânicas, já tiveram seus efeitos anti-AGEs demonstrados tais como *Ilex paraguariensis* A.St.-Hil**.** (BAINS; GUGLIUCCI, 2017)**,** *Eugenia punicifolia* (Kunth) DC (RAMOS *et al.*, 2019) e *Myrcia multiflora* (Lam.) DC (OLIVEIRA *et al.*, 2021). Em Connaraceae para a espécie *C. ferruginea*, no modelo *in vitro*, foi estabelecido que o extrato metanólico das folhas na concentração de 30 µg/mL foi capaz de reduzir em 80% a glicação das hemácias humanas, resultado similar ao efeito demonstrado pelo flavonoide quercetina testado na mesma concentração (ADISA *et al.*, 2004).

Em Connaraceae, diversos táxons já foram estudados quanto ao potencial farmacológico aplicado ao controle glicêmico em abordagens *in vivo* (ADISA *et al.*, 2004, 2010; AKINDELE *et al.*, 2014; DADA *et al.*, 2013; LAIKOWSKI *et al.*, 2017). Para a espécie *C. ferruginea* os autores conseguiram demonstrar efeito hipoglicemiante em ratos cuja diabetes foi induzida pelo agente estreptozotocina (STZ) (ADISA *et al.*, 2010). Laikowski e cols (2017) numa abordagem com *R. cuspidata*, foi demonstraram efeito hipoglicemiante em ratos, no diabetes induzido por STZ, para a fração hidroetanólica (1:1, v/v) do extrato administrado na dose de 200 mg/kg. Com *R. coccinea* foi observado efeito hipoglicemiante no diabetes induzido por aloxano em ratos para a fração hidroetanólica (1:1 v/v) do extrato administrado em diferentes doses compreendidas no intervalo de 100 a 800 mg/kg, em um experimento com duração de 10 dias (DADA *et al.*, 2013). Para a espécie *R. cuspidata*, os autores, utilizando ratos, conseguiram identificar os compostos catequina e/ou seu isômero epicatequina, dois derivados de quercetina (quaijaverina e hiperina) e a proantocianidina A2, sendo esses compostos foram relacionados ao efeito hipoglicemiante observado para esta espécie vegetal (LAIKOWSKI *et al.*, 2017). Em todos esses estudos, os autores buscaram avaliar os efeitos dos extratos frente a redução da

glicemia sem avaliar os possíveis efeitos secundários, como atividade anti-AGEs, inibidora de enzimas que estão envolvidas na metabolização de carboidratos, bem como, a reversão da citotoxicidade celular decorrente da alta concentração de glicose. Assim, com base na composição química até então estabelecida para Connaraceae, cuja presença de polifenois (PIRES *et al.*, 2017), flavonoides (AHMADU *et al.*, 2007; KALEGARI *et al.*, 2014b; LAIKOWSKI *et al.*, 2017) e procianidinas (KALEGARI *et al.*, 2014b; LAIKOWSKI *et al.*, 2017) tem sido relatada, sugerimos que outros efeitos biológicos para além da redução da glicemia possam estar presentes e promover redução das complicações do Diabetes.

3 OBJETIVOS

3.1 Objetivo geral

- Avaliar o perfil químico e o potencial farmacológico frente ao diabetes para 7 diferentes espécies de Connaraceae: *C. blanchetii, C. nodosus, C. regnellii, C. suberosus, R. cuspidata, R. glazioui* e *R. induta*.

3.2 Objetivos específicos

1. Revisar o potencial farmacológico da família Connaraceae e suas possíveis contribuições ao desenvolvimento de novas identidades químicas para uso humano;
2. Comparar métodos diferentes de extração das espécies estudadas quanto ao perfil quantitativo e qualitativo de metabólitos secundários;
3. Estudar a composição de metabólitos secundário através de CL/EM analisando os dados em diferentes plataformas de informática com a finalidade de identificação de compostos;
4. Realizar fracionamento orgânico com diferentes solventes e determinar quantitativamente o teor de polifenóis, flavonoides e taninos em adição a um screening de atividade antioxidante com 2,2-difenil-1-picril-hidrazil (DPPH).
5. Avaliar o perfil antioxidante dos extratos e frações mais ricas em metabólitos secundários a partir dos ensaios de DPPH e *Ferric Reducing Antioxidant Power* (FRAP);
6. Estudar o efeito anti-AGES dos extratos mais ricos em metabólitos secundários no modelo *in vitro* através do estudo pelas vias oxidativa e não oxidativa;
7. Determinar o efeito dos extratos quanto a inibição das enzimas α-amilase e α-glicosidase relacionando essas atividades ao perfil qualitativo de metabólitos secundários.
8. Avaliar a possível toxicidade em células endoteliais (*human umbilical vein cell line*) EA.hy926 bem como a capacidade dos extrato de *Connarus suberosus, Rourea*

cuspidata e *Rourea glazioui* em reverter a citotoxicidade induzida pela hiperglicemia nestas células.

4 RESULTADOS

Os resultados desta tese estão apresentados na forma de quatro capítulos:

- No Capítulo 1 temos o artigo de revisão intitulado: "Connaraceae: *An updated overview of research and the pharmacological potential of 39 species*" publicado na revista *Journal of Ethnopharmacology*, Qualis A1, Impact Fator 4.36.
- No Capítulo 2 há o artigo intitulado: "*Four almost unexplored species of Brazilian Connarus (Connaraceae): Chemical composition by ESI-QTof-MS/MS–GNPS and a pharmacologic potential"* publicado na revista *Phytochemical Analysis*, Qualis A1, Impact Fator 3.37.
- No Capítulo 3 encontra-se o manuscrito intitulado: "*Chemical Composition, Antioxidant and Anti-AGEs Activities in four species of the Connaraceae Family*" o qual será submetido a revista *Journal of Natural Products*, Qualis A1, Impact Fator 4.05.
- No Capítulo 4 são apresentadas a metodologia e resultados referentes aos objetivos específicos 7 e 8 (que comporão um 4º artigo que será posteriormente escrito).

Capítulo 1

Connaraceae: *An updated overview of research and the pharmacological potential of 39 species*

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Connaraceae: An updated overview of research and the pharmacological potential of 39 species

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ABSTRACT

Ethnopharmacological relevance: An interdisciplinary scientific investigation of biologically active agents is fundamental to search for natural substances with therapeutic action. This review collected the most relevant information on traditional knowledge related to the use of plants of the Connaraceae family. This work is the first to compile all the published ethnobotanical, chemical, pharmacological, and toxicological information about this important plant family.

Aim of the study: Our objective was to provide the scientific community with an up-to-date overview of the pharmacological potential of Connaraceae species.

Material and methods: We searched NCBI Pubmed Central, Google Scholar, Scientific Electronic Library Online (SciELO), ScienceDirect, SciFinder, and Scopus databases to review the research on ethnobotanical, chemical, pharmacognostical, pharmacological, and toxicological studies with Connaraceaes. Books that address the theme were also included.

Discussion and conclusion: The literature review indicated that 39 species of Connaraceaes have pharmacological potentiality. Ethnobotany reports listed 36 of the 39 species discussed. Pharmacognostical studies have been conducted with 23 species and isolates, and chemical compounds have been identified for only 15 species. At least one study has been published concerning the pharmacological activities for 20 of the 39 species analyzed. For *Agelaea pentagyna, Cnestis ferruginea, Connars suberosus,* and *Rourea minor*, pharmacological activity experiments were performed using isolated compounds, which have the highest current pharmacological potential. Studies employing a toxicological approach cover only 10 of the 39 Connaraceaes species. Thus, scientific community should conduct much more research for a broader understanding of this plant family.

1. Introduction

Rainforests contain a vast reservoir of plants with associated pharmacological potential, which could provide chemical compounds for the development of new drugs (Ishola et al., 2012a). In this context, the botanical family Connaraceae stands out because it includes many species with high medicinal potential (Akindele and AuthorAnonymous, 2007a; Amos et al., 2002; Ishola et al., 2013a; Kuwabara et al., 2003; Laikowski et al., 2017). Its species grow in tropical areas of South and Central America, Africa, and Asia; the neotropical region is the center of species richness, containing over half of the total number of taxa (Breteler, 1989; Lemmens et al., 2004). In the New World, Connaraceae species are mainly distributed in the Amazon Rainforest and Atlantic Rainforest (Forero, 2002, 1983; 1981; Groppo et al., 2010; Toledo and Souza, 2019).

Different ethnobotanical studies on Connaraceae have encouraged investigation of folk medicine to contribute and facilitate discoveries of bioactive molecules by the scientific community (Longanga et al., 2000a; Soejarto et al., 2012). In addition, numerous plants in the family are used as a source of traditional medicines in different parts of the

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world (Ajibesin et al., 2008; Chifundera, 2001; Choudhury et al., 2015; Ghorbani et al., 2011; Holaly et al., 2015; Lee et al., 2019; Moura et al., 2015; Samuel et al., 2010; Soelberg et al., 2015; Tchicaillat-Landou et al., 2018; Yetein et al., 2013). However, all of these works focus on only one or a few species, and information about this plant family has never been compiled. Based on the literature, the scientific community lacks up-to-date work on the research landscape and pharmacological potential of the family, with only one review focused on a single species (Ha, 2017). Moreover, the study of pharmacological activities in Connaraceae may help to protect biomes (Charneau et al., 2015) because it reinforces the chemical potential of this family and contributes to the development of new drugs.

The pharmacological potential of many species of Connaraceae have been reported in the literature (Ishola et al., 2012c; Kulkarni et al., 2014; Laikowski et al., 2017; Yakubu and Atoyebi, 2018). Some studies have demonstrated relevant pharmacological activities by isolating and characterizing major chemical compounds (Costa et al., 2014; Ishola et al., 2012c; Kuwabara et al., 2003; Laikowski et al., 2017). Some of these species are candidates to provide hypoglycemic medications (Adisa et al., 2010; Dada et al., 2013; Kulkarni et al., 2014; Laikowski et al., 2017), other medications act on the central nervous system (CNS) (Aruoma et al., 2003; Ishola et al., 2013b, 2016, 2012c) or antimicrobial agents (Ahmadu et al., 2006; Bero et al., 2009; Farias et al., 2013; Mesia et al., 2008), while others have various pharmacological effects (Akindele and Adeyemi, 2006; Chung et al., 2009; Reanmongkol et al., 2000). Some authors have described species of Connaraceaes as toxic (Garon et al., 2007; Standley, 1926; Vickery and Vickery, 1974). Rourea coccinea (Schumach. & Thonn.) Benth., Rourea glabra Kunth, and Cnestis ferruginea DC. have pharmacological and toxicological activity reports. However, chemical molecules responsible for each biological action have not been identified, and the safe and toxic dosages need to be established.

The aim of this work is to provide an updated and expanded overview of the ethnobotany use, pharmacological potential, chemical composition, and toxicological profile of Connaraceae species, in order to facilitate understanding of scientific development on this subject. Such information, combined with a discussion on the distribution and number of species in the family, may be used to support future research for drug development related to Connaraceae. Thus, the data presented here may assist work to scientifically confirm ethnobotanical uses that have not been studied yet.

2. Materials and methods

This review includes published scientific papers containing botanical, ethnobotanical, phytochemistry, pharmacological, and toxicological information on Connaraceae species with associated pharmacological potential. This potential is characterized by the description of a particular species in at least one scientific study with ethnobotany approach or report in at least one study about pharmacological activity with application to human health.

NCBI PubMed Central, Google Scholar, Scientific Electronic Library Online (SciELO), ScienceDirect, SciFinder, and Scopus databases were searched for relevant studies. The following descriptors were used: Connaraceae, ethnobotanical, botanical characteristics, chemical composition, pharmacology, and toxicology. The inclusion criteria for articles and books followed the methodology described by (Medeiros et al., 2013) with some modifications. All studies, from anywhere in the world, published up to September 2019 that clearly met the following criteria were included: (1) presents relevant botanical characteristics for species with ethnobotanical or pharmacological potential or describes the geographic location of the species; (2) addresses at least one species of Connaraceae in a study employing ethnobotanical research; (3) describes the qualitative or quantitative profile of secondary metabolites of one or more species in the family; (4) identifies and/or isolates secondary metabolite(s); and (5) includes at least one in vivo or in vitro experiment focusing on the pharmacological and/or toxicological activities of at least one extract obtained from Connaraceae species. Finally, all selected scientific publications were reviewed for acceptability of scientific species names and authors according to Tropicos (http://www.tropicos.org/), The Plant List (http://www.theplantlist.org/), and specific literature for the family (Breteler, 1989; Leenhouts, 1958; Toledo and Souza, 2020). All publications were grouped under the currently accepted scientific name.

Geographic distribution maps presented in this review correspond to the Connaraceae species that have been focus of medicinal and phytochemical studies. The maps were prepared using ArcGIS 10.5 (Environmental Systems Research Institute, 2020), and geographical coordinates were compiled based on information available in herbarium sheet label and specific literature for the concerning genera (Schellenberg, 1938; Leenhouts, 1958; Forero, 1983; Breteler, 1989). In this latter case, geographical points on the maps represent approximate locations.

2.1. Study species

The Connaraceae family can be morphologically recognized by the alternate and compound leaves without stipules, stamens alternating in length, gynoecium with one or five free carpels, and fruits usually in follicles (Forero, 1983; Lemmens et al., 2004). Most species are lianas or scandent shrubs, occasionally treelets, characteristics of low land tropical forest or savannas from Africa, Asia, and South America. Due to the characteristics of the leaves and fruits, the family was once included within the order Sapindales (Candolle, 1825; Jussieu, 1789), but recent phylogenetic studies have proven that Connaraceae belongs to Oxalidales and is a sister group of Oxalidaceae (APG IV, 2016), which includes largely known species, such as the “wood sorrels” and the consumable-fruit species “carambola”. Connaraceae differs from this family by the completely free carpels and follicular fruits.

The currently accepted classification of Connaraceae recognizes 12 genera, which are grouped in four different tribes (Connareae, Jollydoreae, Manoteae, and Cnestideae), according to Lemmens (1989) and Lemmens et al. (2004). The main characteristics used to recognize these tribes and respective genera include: number of leaflets, number of carpels (fiver or one), orientation of sepals on fruits, and presence/absence of endosperm (Lemmens et al., 2004).

Recognition of number of genera and species in Connaraceae have been debated for a long time. While some authors recognized 16 genera and 300–350 species (Forero, 1983; Schellenberg, 1938), others accepted 12 genera and about 200 species (Breteler, 1989; Lemmens et al., 2004). In this review, we follow a more conservative concept, which is presented in the latter two works, although emphasizing that a more comprehensive study on Connaraceae is still needed to determine a more realistic number of species. Moreover, new species of the family have been described in recent years (Morales, 2007; Toledo et al., 2019; Toledo and Souza, 2019, 2018), reinforcing the need for a taxonomic update.

3. Results and discussion

This review discussed 171 publications on Connaraceae, including 154 articles, 12 books, and 5 websites. Analysis about the number of papers found by this study demonstrated that approximately two-thirds of the total research conducted on this family were published in the last decade. This growing interest of the scientific community in this plant family can be gauged by this recent increase in publications. With the information compiled from these works, the following sections will discuss uses in traditional medicine, phytochemistry, chemical composition, and pharmacological studies.

Table 1
Description of Connaraceaes species with reports of ethnobotanical use and/or pharmacological study description with emphasis on human health.

Species	Habit	Native distribution	Vernacular names	Plant part	Traditional uses	References
Agelaea borneensis (Hook.f.) Merr.	Liane	Asia	Akar rusa-rusa	Bark	Inflammatory conditions.	Chung et al. (2009)
Agelaea macrophylla (Zoll.) Leenh.	Liane or shrub	Asia	Akar pinang kutai	Barks, leaves, and roots.	Acne and rheumatisms.	(Samuel et al., 2010; Steenis, 1958)
Agelaea pentagyna (Lam.) Baill.	Liane or shrub	Africa	Ahanhlazu, acroe, Vahimainty, Kanhandi, Vahimenty Rangahtsara, oboki	Bark and leaves	Aphrodisiac, urine retention, diarrhea, general fatigue, stomachache, human salmonellosis, malaria, scabies, and gonorrhea.	(Beaujard, 1988; Obiang et al., 2015; Razafindraibe et al., 2013; Soelberg et al., 2015; Vickery and Vickery, 1974)
Cnestis corniculata Lam.	Liane or shrub	Africa	Kizikizamba, Nyamawa	Roots	Allergic shock, pain, and hemorrhoids.	(Kanteh and Norman, 2015; Lautenschläger et al., 2018; Vickery and Vickery, 1974)
Cnestis ferruginea DC.	Liane or shrub	Africa	Fura amarya; Oen-tolôe, treventi-ito (ba), naporó, nerego, nológo (bj), udju-di-onça, utin ewa, utin ebua, n'konkone, Oko-Aja' or 'Gboyín-Gboyín'	Branches, fruits, leaves, and roots	Diabetes, inflammatory conditions, periodontitis, headache, bronchitis, eye troubles, dysmenorrhea, pains, sinusitis, toothache, conjunctivitis, ear puss, diarrhea, snakebites, and scabies.	(Ajibesin et al., 2008; L. Catarino et al., 2016; Luís Catarino et al., 2016; Diehl et al., 2004; Frazão-Moreira, 2016; Holaly et al., 2015; Houghton and Osibogun, 1993; Ishola et al., 2011; Lautenschläger et al., 2018; Le Grand, 1989; Soelberg et al., 2015)
Cnestis palala (Lour.) Merr.	Liane or shrub	Asia	Terkilir, Binsangut, Maa tai mi-phaak-laak, Belimbing Utan, Kan Yam Dia, Ngonkai; Akar sembelit	Leaves, steam and roots	Fever stomachache, malaria, urinary trouble, anemia in postpartum, bruises, constipation, and snakebites.	(Mahyar et al., 1991; Nordin and Zakaria, 2016; Ong and Nordiana, 1999; Panyaphu et al., 2011; Samuel et al., 2010)
Cnestis polyphylla Lam.	Liane or shrub	Africa	Sefana, Sefa, Sodifafana	Leaves and steam	Diarrhea, fever, malaria, fatigue, and skin injuries.	(Aruoma et al., 2003; Beaujard, 1988; Rakotoarivelo et al., 2015; Rasoanaivo et al., 1992; Riondato et al., 2019)
Connarus africanus Lam.	Liane	Africa	Ganganlisé	Leaves	Hemorrhage.	Allabi et al. (2011)
Connarus angustifolius (Radlk.) G. Schellenb.	Tree	America	Barbatimão do pará, marassacaca, muraçacaca, pajurana, pajujurana, raparigeira, ah-da-ko-de-de-nah, seweyuballi. wah-kah-pwe-muh	Bark	Washing postpartum, genitourinary infections, uterus problems, ovarian cysts, vaginal discharge, stomachache, malaria, and headache.	(Coelho-Ferreira, 2009; DeFilipps et al., 2004; Paracampo, 2011; Pastore et al., 2017; Pires et al., 2017; Suffredini et al., 2007; Yazbek et al., 2016)
Connarus cochinchinensis (Baill.) Pierre	Not described	Asia	Sembilat	Leaves	Tuberculosis.	Zarr (2010)
Connarus detersus Planch.	Tree	America	Cabelo de Negro	***	***	Farias et al. (2013)
Connarus erianthus Benth. ex Baker	Bush or treelet	America	Tala	Stem-bark and root-bark	Menstrual bleeding.	(DeFilipps et al., 2004; Fern et al., 2014)
Connarus favosus Planch.	Bush or liane	America	Veronica	Bark	Snakebites.	(Gombeau et al., 2019; Moura et al., 2015; Silva et al., 2016)
Connarus lambertii (DC.) Sagot	Bush or liane	America	Tuktuk	Bark and leaves	Diarrhea and as an astringent.	(Coe et al., 2010; Jiménez et al., 2001)
Connarus monocarpus L.	Tree	Asia	Agil	Roots	Snakebites.	(Aiyar et al., 1964; Dharmadasa et al., 2016)
Connarus paniculatus Roxb.	Tree	Asia	Uroshichak	Leaves	Stomach and diarrhea.	(Choudhury et al., 2015; Henkin et al., 2017; Le et al., 2005; Roy Choudhury et al., 2015)
Connarus patrisii (DC.) Planch.	Bush or liane	America		Seeds	Recovering strength after illness.	Fern et al. (2014)
Connarus planchonianus G. Schellenb.	Liane	Asia	Sembelit angin	Stems and roots	Flatulence.	Ong and Nordiana (1999)
Connarus punctatus Planch.	Bush or liane	America		Stem-bark and root-bark	Menstrual bleeding.	(DeFilipps et al., 2004; Fern et al., 2014)
Connarus ruber (Poepp.) Planch.	Bush or liane	America	Pitso tapa	Fruit and leaves	Diabetes.	(Castilho et al., 2014; Paniagua Zambrana et al., 2017; Pastore et al., 2017)
Connarus semidecandrus Jack	Liane	Asia	Đho Rđo, Bob Jei Pei	Leaves, steam, and roots	Diarrhea and fever.	(Lee et al., 2019; Reanmongkol et al., 2003; Srithi et al., 2009)
Connarus suberosus Planch.	Bush or tree	America	Tropeiro ou bico de papagaio, galinha-choca	Bark	Diarrhea and heart problems.	(Charneau et al., 2015; Costa et al., 2014; Taveira et al., 1988)
Manotes expansa Sol. ex Planch.	Liane	Africa	Menga-menga, M'memenga	Leaves	Hypertension, hemorrhoids, bleeding, stomach pains, scoliosis, dysentery, and ophthalmic problems.	(Lautenschläger et al., 2018; Makambila-Koubemba et al., 2011; Malan et al., 2015; Mesia et al., 2008; Tchicaillat-Landou et al., 2018)
Pseudoconnarus macrophyllus (Poepp.) Radlk.	Liane	America	***	***	***	Suffredini et al. (2007)

(*continued on next page*)

Table 1 (*continued*)

Species	Habit	Native distribution	Vernacular names	Plant part	Traditional uses	References
Pseudoconnarus rhynchosioides (Standl.) Prance	Liane	America	Saracura	Bark	Exhaustion, sexual stimulant, and malaria.	Pedrollo et al. (2016)
Rourea acutipetala Miq.	Liane	Asia		Roots	Lumbago.	Fern et al. (2014)
Rourea coccinea (Schumach. & Thonn.) Benth.	Liane	Africa and Asia	Ploem-tjo, sjo-tami (for the fruit); Tsaamiyar-kasa; Oke abolo; Orikoteni; Amuje; Ganganlissè; Vikplomba	Whole plant	Jaundice, venereal diseases, as a sedative, diarrhea, inflammation, urogenital diseases, urinary problems, tumors, earache, muscular, human salmonellosis, malaria, rheumatic pains, breast milk enhancement, anti-cancer activity, and high blood pressure.	(Ahmadu et al., 2007; Akindele and AuthorAnonymous, 2007b; Akindele et al., 2014; Bero et al., 2009; Chhabra et al., 1993; Dougnon et al., 2017; Kankara et al., 2015; Soelberg et al., 2015; Soladoye et al., 2010; Yetein et al., 2013)
Rourea cuspidata Benth ex. Baker	Liane	America	Cipo miraruina	Bark	Diabetes.	Laikowski et al. (2017)
Rourea doniana Baker	Liane	America	***	***	***	(Oliveira et al., 2012, 2010)
Rourea emarginata (Jack) Jongkind	Liane	Asia		Leaves	Fever, aches, and sores.	Fern et al. (2014)
Rourea fulgens Planch.	Liane or shrub	Asia		Leaves and roots	Stomachache.	Fern et al. (2014)
Rourea glabra Kunth	Shrub	America		Seeds	Cutaneous diseases.	(Fern et al., 2014; Standley, 1926)
Rourea induta Planch.	Shrub	America	Hohocré, chapeudinha, pau- de-porco or campeira	Leaves and roots	Rheumatisms, Chagas diseases, and as an abortive.	(Kalegari et al., 2014a, 2011; Rodrigues, 2007; Yazbek et al., 2016)
Rourea mimosoides (Vahl) Planch.	Liane	Asia	Sembelit merah darah	Roots	Cough with blood, bloody diarrhea, and diuretic.	(Grosvenor et al., 1995; Sabran et al., 2016)
Rourea minor (Gaertn.) Alston	Liane or shrub	Africa and Asia	Ha ji me wo, Beririt, Akar Kayu Mengecut; Akar sekecut, Anone-lou-chari, hrung-mung.	Leaves and roots	Abrasions, diabetes, lesions, dengue, kidney stones, polio, fever, kidney stone, diuretic, dysentery, bile diseases, uterine contraction stimulant skin inflammation, menstrual bleeding, and wounds.	(Alam, 1992; Ghorbani et al., 2011; Gyllenhaal et al., 2012; Henkin et al., 2017; Jamal et al., 2011; Kong et al., 2008; Kulkarni et al., 2014; Mahyar et al., 1991; NguyêñXuãn Duñg and kDôTâí;Li, 1991; Nordin and Zakaria, 2016; Soejarto et al., 2012; Soro et al., 2012)
Rourea obliquifoliolata Gilg	Liane or shrub	Africa	Lungagula	Root bark	Diarrhea, dysentery, toothache, and elephantiasis.	(Longanga et al., 2001, 2000a; 2000b)
Rourea orientalis Baill.	Shrub or small tree	Africa	Kazingini, munhadozwarozwa, sambaucaranga, muziriri, Hombo-kisogo, Kisogo, Liyenzi; Msogo, Mhombo.	Stems, bark, leaves and roots	Diarrhea, antiemetic, menstrual troubles, headache, fever, and skin injuries.	(Bruschi et al., 2011; Chhabra et al., 1993; Chinsembu, 2016; Fern et al., 2014; Jeannoda et al., 1985; Ruffo et al., 2002)
Rourea puberula Baker	Shrub or small tree	America	Murku waska	Stems	Health tonic, broken bones, leishmaniasis, rheumatism, and vaginal pains.	Sanz-Biset and Cañigueral (2011)
Rourea thomsonii (Baker) Jongkind	Liane or shrub	Africa	Lwamba	Bark	laxative and constipation.	Chifundera (2001)

3.1. Traditional uses

This review found that 36 of the 39 Connaraceae species with associated pharmacological potential have traditional medicinal use in tropical areas worldwide (Table 1). The traditional uses reported in the different ethnobotanical studies include a wide range of therapeutic functions, which can guide research for actions that have not been confirmed yet by the scientific community. Additionally, popular names can assist researchers to obtain samples at the locations where each species can be found. Moreover, we indicate which part of the plant is traditionally used to ensure that future research can properly select the plant organ to study.

Of the approximately 200 recognized Connaraceae species (Lemmens et al., 2004), 39 or about 20% are known to have potential pharmacological activities. This relatively low percentage of species studied suggests that the huge pharmacological potential for Connaraceae has only been superficially explored by the scientific community and additional research should be undertaken.

Moreover, this review verified that among the 12 recognized genera of Connaraceae, only six (*Agelaea* Sol. ex Planch., *Cnestis* Juss., *Connarus* L., *Manotes* Sol. ex Planch., *Pseudoconnarus* Radlk., and *Rourea* Aubl.) have reported ethnobotany use or were the focus of pharmacological investigation. Consequently, half of the genera have not yet been tested for phytochemical and/or pharmacological uses. Of the six genera reported for medicinal use, we observed that *Agelaea, Cnestis, Connarus, Manotes, Pseudoconnarus,* and *Rourea* present three, four, fifteen, one, two, and fourteen species, respectively.

Agelaea is found in central Africa and southeastern Asia, and the three species reported here are distributed in central Africa, Madagascar, and from central Vietnam to Java (Fig. 1). The species of *Cnestis* listed in this review have similar distribution as those of *Agelaea*, although *Cnestis polyphylla* extends a little further south in Africa (Fig. 2). *Connarus*, on the other hand, is the largest genus of the family and has a pantropical distribution, with the species of medicinal interest mainly occurring in the Amazonian region of South America as well as in western Africa and from northern India to Java (Fig. 3). *Manotes* is exclusively found in Africa, and the only species reported here is distributed in western and central Africa (Fig. 4). *Pseudoconnarus* is only found in South America and the two species studied in the medicine occur in the Amazon forest, with *P. macrophyllus* being widely distributed in this region (Fig. 4). Finally, *Rourea* is a pantropical genus, with the species of medicinal interest investigated so far occurring from southern Mexico to southeastern Brazil in the Neotropics, western, central, and southeastern Africa, Madagascar, and from western Myanmar to Java, with few records of *Rourea minor* being found in northern Australia and New Caledonia and adjacent islands (Fig. 5).

Of the 39 species investigated in this review, 16 occur in the Americans, 11 in Africa and Madagascar, and 14 in Asia. Only *Rourea coccinea* and *Rourea minor* are found in the latter two continents. Most of these species are widely distributed, although *Pseudoconnarus rhyncosioides,* and *Rourea fulgens* apparently have restricted distributions, which demands special attention while collecting individuals/samples for phytochemical studies to ensure sustainable use and their preservation, as well as the ecosystems they inhabit.

3.2. Phytochemistry

The distribution of secondary metabolites for the Connaraceae family is as varied as the phytochemistry classification of the already identified secondary metabolites (Table 2).

For 23 of the 39 species listed in this review, at least one group of constituents has been identified; therefore, for the other 16 species, phytoconstituents are not yet known. Isolation and identification of chemical constituents was observed for only 15 of the 39 Connaraceae species reported in this article. This reinforces the necessity for qualitative and quantitative studies that profile compounds to broaden knowledge about these phytoconstituents and confirm or reject the pharmacological activities described by popular use. Additionally, new studies employing analytical devices and metabolomics tools may contribute to address questions on the basis of taxonomy, facilitating the distinction of species and genera, also valuable to accurately establish the number of recognized taxa in this plant family.

3.2.1. Agelaea

For the genus *Agelaea*, two of the three species with associated pharmacological potential have identified chemical compounds (Fig. 6 – Supplementary files). Essential oils, coumarins, and just one flavonoid has been were isolated and characterized in *Agelaea*. For *Agelaea borneensis*, the study conducted by Sardans et al. (2015) identified monoterpenes (limonene and α-pinene) and sesquiterpenes (β-caryophyllene and D-germacrene). For *Agelaea pentagyna,* the presence of coumarin compounds o-coumaric acid, 4-hydroxycoumarin, and dicumarol was reported (Vickery and Vickery, 1980), and flavonoid tricin was isolated from methanolic extract in *A. pentagyna* leaves (Kuwabara et al., 2003).

Although the literature reports that the chemical compounds belong to the alkaloids, phenols and saponins classes in *A. pentagyna* (Obiang et al., 2015) have not been qualitatively described yet.

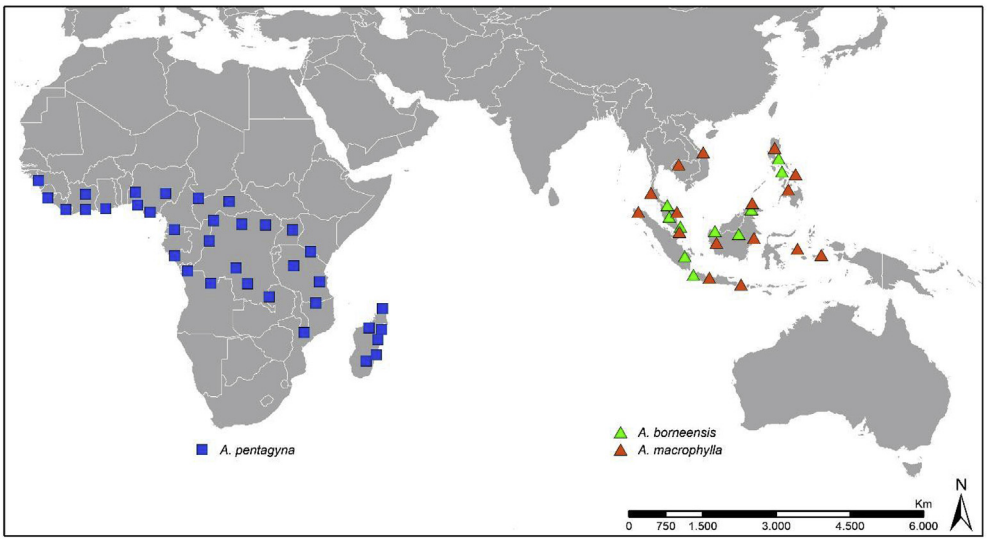

Fig. 1. Distribution of Agelaea species with pharmacological potential.

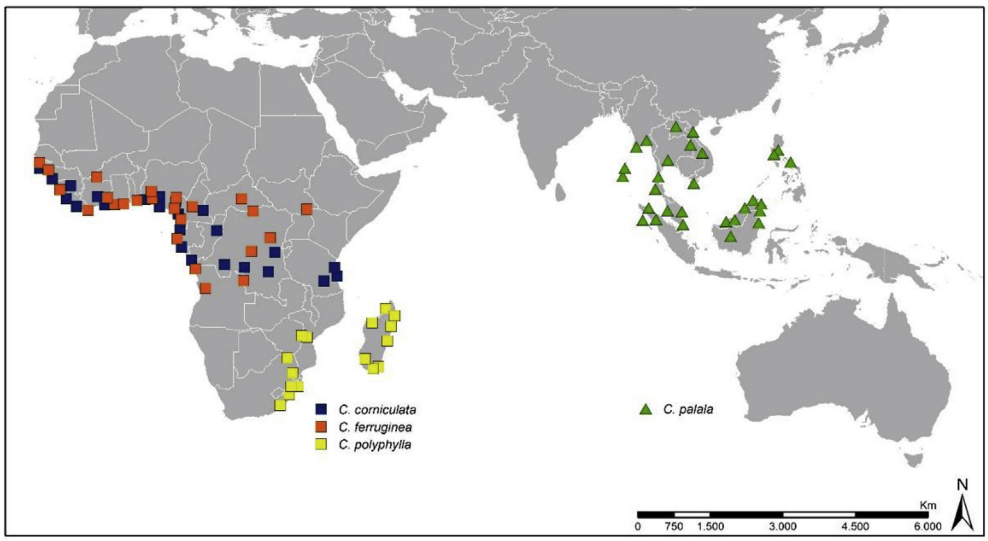

Fig. 2. Distribution of Cnestis species with pharmacological potential.

3.2.2. Cnestis

For the genus *Cnestis*, at least one isolated and identified chemical compound were found in *Cnestis ferruginea*, *Cnestis palala*, and *Cnestis polyphylla*. Different classes of compounds are present in this genus (Fig. 7 – Supplementary files).

Bioflavonoid amentoflavone, an apigenin dimmer, has been reported for methanolic extract of *Cnestis ferruginea* roots (Ishola et al., 2012c). Hydroquinone and robustaside B were isolated in the leaves of this species (Adisa et al., 2011; Adisa and Olorunsogo, 2013). The compounds β-sitosterol and stigmasterol were identified in the ethanolic extract of the roots (Ojo et al., 2019), and β-sitosterol in the leaves of the same taxa (Olugbade et al., 1982). Caffeic acid methyl ester and hydroquinone were isolated from *Cnestis ferruginea* leaves (Kouakou et al., 2019). An evaluation of the phytochemistry composition found in different parts of *Cnestis palala* isolated hydroquinone, β-sitosterol, β-sitosterol-glucoside (daucosterin), ethyl caffeate, scopoletin, and 2 nonenal (Dej-adisai et al., 2015). In another study with *Cnestis palala*, the presence of the compound L-methionine sulfoximine (MSX) was reported from the ethanolic extract of seeds, pods, petioles, roots, and stems, but this was not found in the leaves of this plant (Murakoshi et al., 1993). The unusual amino acid MSX is classified as very toxic (Murakoshi et al., 1993). Thus, the lack of this metabolite in plant leaves is in accordance with the traditional use of this part of the plant, reported in the ethnobotanical studies, which describe the use of leaf for *Cnestis palala* (Mahyar et al., 1991; Nordin and Zakaria, 2016; Samuel et al., 2010). Glabrin has been isolated from the roots of *Cnestis polyphylla* (Jeannoda et al., 1984), and is related to the neurotoxic effect of this plant.

3.2.3. Connarus

The five species of *Connarus* that contain compounds isolated and identified (Fig. 8 – Supplementary files) by the scientific community include *Connarus angustifolius*, *Connarus monocarpus*, *Connarus paniculatus*, *Connarus semidecandrus,* and *Connarus suberosus.*

From the barks of *Connarus angustifolius*, Pires et al. (2017) isolated three phenol gallic, ferulic, and caffeic acid; three flavonoids o-heterosidic, catechin, rutin, and quercitrin; and one phytoalexin resveratrol. Rapanone, bergenin, and leucopelargonidin compounds were isolated from *Connarus monocarpus* roots (Aiyar et al., 1964). Six alkaloids with quinolizidine nucleus, piptanthine, 18-epipiptanthine, ormosanine, homoormosanine, podopetaline, and homopodopetaline were isolated from the fruits and leaves of *Connarus paniculatus* (Le et al.,

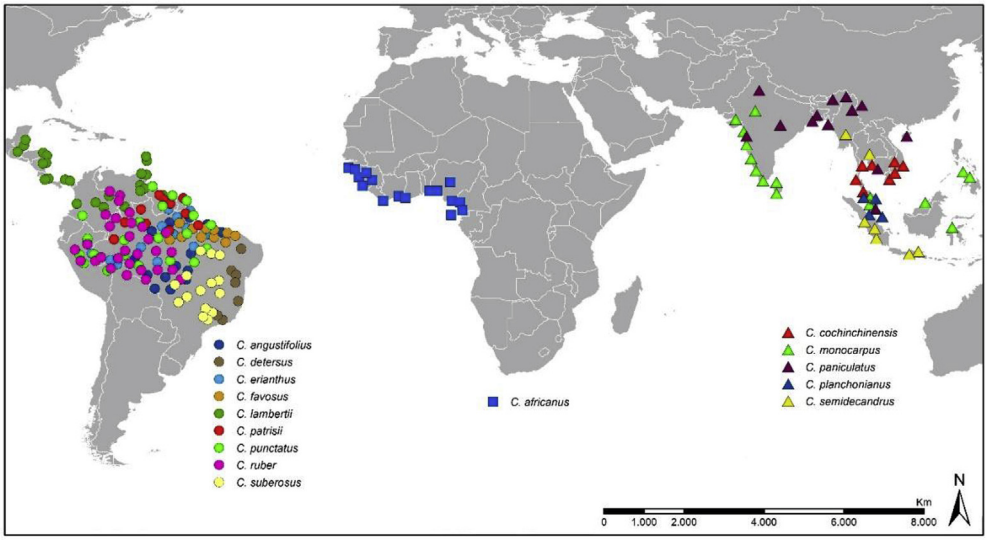

Fig. 3. Distribution of Connarus species with pharmacological potential.

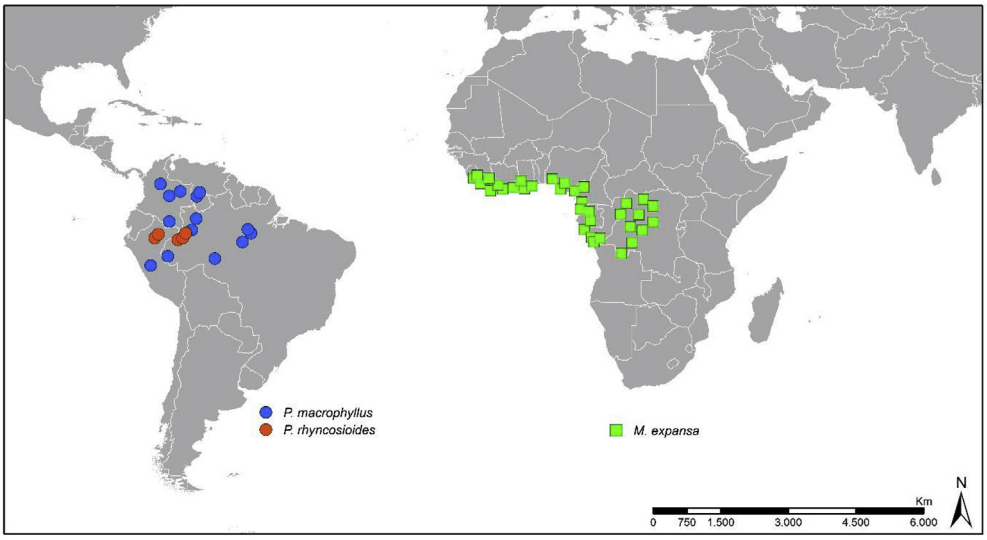

Fig. 4. Distribution of *Manotes and Pseudoconnarus* species with pharmacological potential.

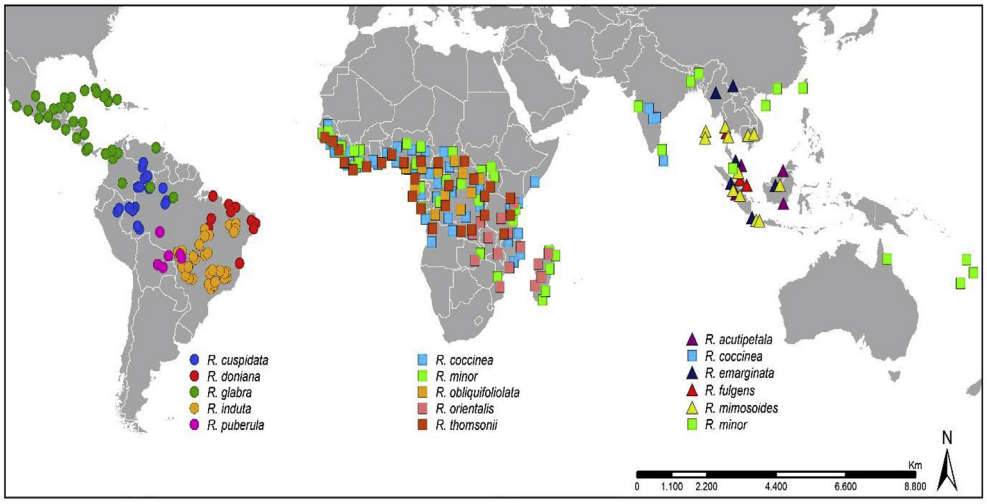

Fig. 5. Distribution of Rourea species with pharmacological potential.

2005). The presence of suberonone, rapanone, β-sitosterol, oleic acid, and geranylgeraniol compounds was reported for extracts of *Connarus suberosus* barks (Costa et al., 2014). Different authors have reported the presence of alkaloids in Connaraceae (Akindele and AuthorAnonymous, 2007b; Farias et al., 2013; Ishola et al., 2011); however, Le et al. (2005) have been the only researchers to successfully isolate and identify such compounds from any of these species.

3.2.4. Manotes and pseudoconarus

No studies have been published employing a qualitative and/or quantitative approach to elucidate the chemical compounds in the genera *Manotes* and *Pseudoconanrus*.

3.2.5. Rourea

The genus *Rourea* contains five plant species (*R. coccinea, R. doniana, R. cuspidata, R. induta,* and *R. minor*) with isolated and identified chemical compounds (Fig. 9a and b – Supplementary files).

From the leaves of *R. coccinea*, Ahmadu et al. (2007) isolated an aglycone flavonoid called quercetin and two O-glycoside-type heterosides identified as isoquercetin and guaiaverin. Another study using the same species reported the presence of chlorogenic acid, rutin, and quercetin for the extract obtained also from the leaves (Akindele et al., 2014). From the leaves and branches of *R. doniana*, Oliveira et al. (2012) isolated five triterpenes (lupeol, lupenone, α-amyrenone, β-amyrenone, and taraxerol), one flavonol (7,4'-dimethylkaempferol), one coumarin (scopoletin), and four phytosteroids (β-sitosterol, stigmasterol, daucosterol and stigmasterol glucoside). In *R. cuspidata*, six chemical compounds have been identified (Laikowski et al., 2017): a ketone called zingerone, a stearic acid-derived amide known as octadecanamide; an aglycone epicatechin flavonoid or its catechin isomer; two O-glycoside flavonoids called guaijaverin and hyperin; and an anthocyanidin called proanthocyanidin A2. A study identified in the leaves of *R. induta* two phenols chlorogenic and neochlorogenic acid, four flavonoids: hyperin, reynoutrin, avicularin and quercetin; as well one anthocyanidin proanthocyanidin C1 (Kalegari et al., 2014a). The leaves and branches of *R. minor* contain quercetrin, hyperin, quercetin, astilbin; two glycosydes: β-sitosterol and β-sitosteryl-β-D-glucopyranoside; and the anthraquinone compounds: physcion and erythroglaucin (Jiang Jian-qin, 1990). In the stems of *R. minor,* 23 compounds were identified, including: bergenin, catechin derivatives, procyanidin A1, scopoletin, fulgidic and pinellic acid (Ngoc et al., 2019). Additionally, He et al. (2006) isolated two components identified as rourinoside and rouremin, obtained from the stem bark of this plant. The rapanone and leucopelargonidin compounds were isolated from *R. minor* roots (Ramiah et al., 1976).

Table 2
Secondary metabolite classes reported for Connaraceae.

Species	Phytochemistry class	References
Agelaea borneensis	Volatile oils	Sardans et al. (2015)
Agelaea macrophylla	NS	
Agelaea pentagyna	Alkaloids, coumarins, flavonoids, and phenols, saponins	(Kuwabara et al., 2003; Obiang et al., 2015; Vickery and Vickery, 1980)
Cnestis corniculata	Coumarins	Vickery and Vickery (1980)
Cnestis ferruginea	Alkaloids, anthraquinones, coumarins, flavonoids, glycosides cardiotonics, phenols, reducing sugars, saponins, sterols, tannins, and volatile oils	(Ishola et al., 2011; Ogunwande et al., 2013; Olugbade et al., 1982; Vickery and Vickery, 1980)
Cnestis palala	Coumarins, glycosidic, volatile oils	Dej-adisai et al. (2015)
Cnestis polyphylla	Flavonoids, proanthocyanidins, saponins, and tannins	Aruoma et al. (2003)
Connarus africanus	NS	
Connarus angustifolius	Coumarins, flavonoids, glycosides cardiotonics, phenols, quinones, saponins, and tannins	(DeFilipps et al., 2004; Pires et al., 2016, 2017)
Connarus cochinchinensis	Saponins	Zarr (2010)
Connarus detersus	Alkaloids, flavonoids, phenols, saponins, and tannins	Farias et al. (2013)
Connarus erianthus	NS	
Connarus favosus	Coumarins, flavonoids, leucoanthocyanidins, phenols, and tannins	Silva et al. (2016)
Connarus lambertii	Alkaloids, flavonoids, and volatile oils	(Coe et al., 2010; Jiménez et al., 2001)
Connarus monocarpus	Anthocyanidins, coumarins, and phenols	Aiyar et al. (1964)
Connarus paniculatus	Alkaloids	Le et al. (2005)
Connarus patrisii	NS	
Connarus planchonianus	NS	
Connarus punctatus	NS	
Connarus ruber	Flavonoids and tannins	Nakamura et al. (2011)
Connarus semidecandrus	Coumarins	Reanmongkol et al. (2003)
Connarus suberosus	Saponins and tannins	Worthley and Schott (1969)
Manotes expansa	Anthraquinones, coumarins, flavonoids, saponins, tannins, and volatile oils	Makambila-Koubemba et al. (2011)
Pseudoconnarus macrophyllus	NS	
Pseudoconnarus rhynchosioides	NS	
Rourea acutipetala	NS	
Rourea coccinea	Alkaloids, anthraquinones, coumarins, flavonoids, glycosidic, phenols, saponins, sterols, tannins, and volatile oils	(Akindele et al., 2014; Vickery and Vickery, 1980; Yakubu and Atoyebi, 2018)
Rourea cuspidata	Anthocyanidins, flavonoids, and phenols	Laikowski et al. (2017)
Rourea doniana	Coumarins, flavonoids, and volatile oils	Pires et al. (2017)
Rourea emarginata	NS	
Rourea fulgens	NS	
Rourea glabra	NS	
Rourea induta	Flavonoids, phenols, and proanthocyanidins	Kalegari et al. (2014a)
Rourea mimosoides	NS	
Rourea minor	Alkaloids, anthraquinones, coumarins, flavonoids, tannins, and volatile oils	(Huo et al., 2011; Jiang Jian-qin, 1990; Kulkarni et al., 2014; Mali and Borges, 2003)
Rourea obliquifoliolata	Flavonoids, saponins, reducing sugars, tannins, and volatile oils	Longanga et al. (2000b)
Rourea orientalis	NS	
Rourea puberula	NS	
Rourea thomsonii	NS	

NS = Not studied.

3.3. Pharmacological activities

The main pharmacological activities reported in studies containing extracts and/or isolated substances conducted in vivo and in vitro with Connaraceaes are presented in this section.

3.3.1. Antipyretic activity

Antipyretic activity has been reported for methanolic extract obtained from *Connarus semidecandrus* roots; when administered to mice at different dosages between 100 and 400 mg/kg, the extract reduced yeast-induced fever (Reanmongkol et al., 2000). A similar effect was observed for *R. coccinea* when the aqueous extract of leaves produced an antipyretic effect in rats at doses of 100, 200, and 400 mg/kg. Those authors verified the antipyretic effect through yeast and amphetamine induced hyperemia models and attributed the effect to a possible inhibition of prostaglandin production promoted by the extract (Akindele and AuthorAnonymous, 2007a).

3.3.2. Anti-inflammatory activity

Methanolic extract obtained from *A. borneensis* bark showed anti-inflammatory activity in vitro through lipoxygenase inhibition mechanism, IC_{50}% 1.6 μg/mL. The authors attribute the anti-inflammatory activity of this plant to the presence of flavonoids and terpenoids (Chung et al., 2009). For the genus *Cnestis*, methanolic extracts obtained from *Cnestis ferruginea* roots demonstrated an anti-inflammatory effect on xylene-induced and formaldehyde assays in rats at doses starting at 200 mg/kg (Ishola et al., 2011). Another bioguided study with *Cnestis ferruginea* associated the anti-inflammatory effect with biflavonoid amentoflavone isolated from this species (Ishola et al., 2012a). In this study, the richest fraction of biflavonoid had the highest anti-inflammatory effect of those tested, indicating that such effect can be associated with this compound (Ishola et al., 2012a). Another study demonstrated the ability of methanolic extract to decrease nitrite release, mitigate free radical generation, and reduce malondialdehyde formation in Rat C6 glioma cell line treated with lypopolysaccharide (LPS) (Ishola et al., 2013a). The authors observed increased glutathione level (GSH) and reduced expression of tumor necrosis factor-α (TNF-α), attributing this effect to the presence of the amentoflavone compound in the fraction of the studied extract (Ishola et al., 2013a). Ethanolic extract from *Cnestis ferruginea* roots reduced cyclooxygenase-2 (COX-2) expression following exposure of mice to kainic acid; the extract administered at 400 mg/kg was able to reduce enzyme expression in the hippocampus reducing neuroinflammation in these animals (Ojo et al., 2019).

In *R. coccinea*, aqueous extract obtained from the leaves, when administered to rats at doses of 100, 200, and 400 mg/kg, demonstrated

anti-inflammatory effect through inhibition of phospholipase A2 or inhibition of cyclooxygenases. This indicates the need to isolate and identify the chemical compounds responsible for the anti-inflammatory effect (Akindele and AuthorAnonymous, 2007b).

3.3.3. Analgesic activity

Methanolic extracts obtained from *Cnestis ferruginea* roots demonstrated analgesic effect in rats, possibly mediated through peripheral and central mechanisms by inhibiting release of histamine, serotonin, and prostaglandins (Ishola et al., 2011). The bark extract of *Connarus suberosus* exhibited an analgesic effect in mice at the dose of 100 mg/kg in test on the reaction time to thermal stimulus (Taveira et al., 1988). The aqueous extract of *M. expansa* leaves presented a significant analgesic effect to different pain models in mice treated with 250 mg/kg of aqueous extract (Makambila-Koubemba et al., 2011). The aqueous extract of *R. induta* leaves administered at doses of 30–300 mg/kg in mice had an analgesic effect, which was verified by different pain measurement experiments, and appears to inhibit the release of inflammatory cytokines, mediated by quercetin-derived flavonoids including the hyperin compound (Kalegari et al., 2014a).

3.3.4. Antihistamine activity

In vitro tests using rat basophilic leukemia cells (RBL-2H3) indicated that flavonoid tricin isolated from *A. pentagyna* leaves had antihistamine activity, in which degranulation inhibition assay presented IC_{50}% 4.83 μm against β-hexosaminidase. According to the authors, flavonoid produced a potent antihistamine release (Kuwabara et al., 2003).

3.3.5. Anticholinesterase activity

Studies with methanolic extract of *Cnestis ferruginea* roots demonstrated anticholinesterase activity in scopolamine-treated mice, in which the acetylcholinesterase enzyme activity reduced with the plant extract tested at doses of 100 and 200 mg/kg and for the amentoflavone sub-fraction at doses of 12.5 and 25 mg/kg, suggesting that this compound is responsible for the observed effect (Ishola et al., 2013b). At a concentration of 5 mg/mL, an ethanolic extract of from *Connarus detersus* seeds promoted a 91.9% inhibition of acetylcholinesterase enzyme activity; however, the researchers did not isolate and identify the compounds responsible for this action (Farias et al., 2013).

3.3.6. Anticonvulsant activity

In vivo studies from ethanolic extract of *Cnestis ferruginea* roots found an antiepileptic effect in mice with kainic acid-induced epilepsy. The authors demonstrated that the anticonvulsant effect of the extract administered orally at a dose of 400 mg/kg was related to reducing the activity of neuroinflammation and oxidative stress in the hippocampus of these animals (Ojo et al., 2019).

3.3.7. Neuroprotective activity

In vivo studies using methanolic extract of *Cnestis ferruginea* roots evidenced memory protective activity in mice, which had improved locomotion patterns and neuroprotective effect (Ishola et al., 2013b). The observed effects produced by the extract and the isolated compound indicate that both compounds may be candidates for neuroprotective medications. The aqueous extract of *Cnestis ferruginea* fruits showed GABAergic activity in rats with ketamine-induced psychosis; when administered for 7 day at a dose of 2 g/kg, the extract increased the gamma aminobutyric acid (GABA) levels in the nervous system of these animals (Ebuehi and Aleshinloye, 2010).

3.3.8. Antidepressant and anxiolytic activity

An in vivo study using methanolic extract of *Cnestis ferruginea* roots evidenced an anxiolytic and antidepressant effect in mice treated with cyproheptadine a 5HT2 receptor antagonist at doses ranging from 25 to 200 mg/kg (Ishola et al., 2012c). That study also demonstrated anxiolytic and antidepressant effect for an amentoflavone compound isolated from this plant extract administered at doses of 6.25–50 mg/kg. According to the study, which used different agonist and antagonist substances from different receptors, these effects are apparently mediated through the involvement of GABAergic, noradrenergic, and serotonergic systems and present a favorable safety profile for the development of human medications (Ishola et al., 2012c).

3.3.9. Antioxidant activity and chemical evaluation of radical scavenging

The ethanolic extract of *A. pentagyna* leaves exhibited activity in an 2,2-diphenyl-1-picrilhydraz (DPPH) assay, with an IC_{50} of 177.02 μg/mL. The authors stated that this potential effect is related to the presence of saponins, flavonoids, and phenols present in secondary metabolites of this plant (Obiang et al., 2015).

The antioxidant potential in *Cnestis ferruginea* has been demonstrated in several studies (Adisa et al., 2011; Adisa and Olorunsogo, 2013; Ojo et al., 2019). Fractionated extracts of *Cnestis ferruginea* demonstrated antioxidant action and elimination of free radical by various extracts and sub-fractions in different vitro assays, including superoxide anion scanning, DPPH, inhibition of xanthine oxidase activity, and inhibition of Fe2 +/ascorbate (Adisa et al., 2011). Another study using the same plant species indicated potent antioxidant action for the robustaside B and para-hydroxyphenol isolated substances obtained from the leaves (Adisa and Olorunsogo, 2013). The compounds were incubated at different concentrations (0.05–1 mM) with mitochondria obtained from rat-liver tissue, and a significant reduction of thiobarbituric acid reactive substances (TBARS) was observed in a concentration-dependent manner (Adisa and Olorunsogo, 2013). Using a different approach, ethanolic extract of *Cnestis ferruginea* attenuated oxidative stress induced by kainic acid. In this study, the 400 mg/kg extract promoted increased glutathione (GSH), reduced nitrite and MDA production, and attenuate TNF-α mediated signaling (Ojo et al., 2019). In addition, methanolic extract of *Cnestis ferruginea* roots tested at 100 and 200 mg/kg, as well as the sub-fraction rich in amentoflavone administered at 12.5 and 25 mg/kg, reduced the production of malondialdehyde and increased GSH levels in mice (Ishola et al., 2013b).

The aqueous extract of *Connarus favosus* bark at a concentration of 5 mg/mL exhibited activity in the DPPH and Fe^{3+}/Phenanthroline in vitro screening assays with 4.0 ± 0.3 and 1.7 ± 0.3 equivalents of ascorbic acid [AAeq]. The extracts with AAeq value between 1.0 and 2.0 exhibited a high scavenging activity, which was demonstrated by the DPPH assay. This result is associated with the presence of phenolic compounds in the metabolites of this plant (Silva et al., 2016). An evaluation of the methanolic extract of *Connarus paniculatus* branches for the inhibitory activity of glycation and product formation demonstrated that this species could inhibit formation of these products, with an IC_{50} of 2.75 ± 0.31 μg/mL (Tran, 2015). The aqueous extract of *Connarus angustifolius* bark (10% w/v) and the ethanolic, butanolic, and ethy acetate fractions (5% w/v) eliminated the DPPH radical above 70%. This antioxidant action is credited to the presence of flavonoids catechin and rutin isolated from the extracts of this plant, which is widely used in traditional Brazilian medicine (Müller et al., 2016). The extract obtained from *Connarus ruber* barks at a concentration of 200 ppm inhibited 80% of the DDPH radical (Nakamura et al., 2011).

The hydroethanolic extract of *R. coccinea* leaves presented favorable antioxidant profile in rats treated with the extract at doses of 100, 200, and 400 mg/kg (Akindele et al., 2014). These researchers observed increased catalase (CAT), superoxide dismutase (SOD), GSH, and glutathione peroxidase (GPX) activity, as well as a reduced malondialdehyde levels, measured in the cardiac tissues of these animals attributed to flavonoids and other chemical constituents in the plant extract (Akindele et al., 2014). Another study with the same species demonstrated in vivo antioxidant effect for an aqueous extract of leaves on isoniazid-induced oxidative stress in rats (Andrew et al., 2017). In this study, there was a quantitative reduction in serum catalase enzyme units at a dose of 200 mg/kg. In carbon tetrachloride (CCl4) induced

oxidative stress, the aqueous extract of the leaves was equally promising. Administered for seven days concomitantly with CCl_4, the extract at doses of 200, 400, and 1.000 mg/kg quantitatively increased CAT, SOD, and hepatic peroxidase enzymes, reduced glutathione, as well as being able to decrease malondialdehyde (Akindele et al., 2010).

The ethanolic extract of *R. induta* leaves administered to rats at dose 500 mg/kg had an antioxidant effect after induction of oxidative stress by carbon tetrachloride. This study also demonstrated that the extract could restore CAT, GPx, SOD, and GSH antioxidant defenses as well as reduce TBARS levels, which were attributed to the presence of hyperin, quercetin-3-O-α-arabinofuranoside (avicularin) and quercetin-3-O-β xyloside (reynoutrin) (Kalegari et al., 2014b). Essential oils obtained from the leaves of *R. minor* eliminated DPPH and 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) $ABTS^{\cdot+}$ radicals due to the presence of oxygenated compounds among the secondary metabolites isolated in this plant (Huo et al., 2011).

The aqueous extract of *Connarus favosus* barks showed protective activity against mercury poisoning in the in vivo model with zebrafish (*Danio rerio*). This study demonstrated the activity of the 5% extract added to their food, which was administered to animals at a dose of 250 mg/kg over a period of 60 days (Gombeau et al., 2019). The observed effects include reduced lipid peroxidation among animals exposed to mercury and supplemented by the extract compared to animals only exposed to the metal (Gombeau et al., 2019). According to the authors, the observed effect is derived from the reduction of mercury-induced lipid peroxidation at the brain and liver levels by this plant in the face of exposure to heavy metals, which is mediated by antioxidant mechanisms.

3.3.10. Hypoglycemic activity

Methanolic and ethyl acetate extracts obtained from *Cnestis ferruginea* leaves exhibited a hypoglycemic effect at the dose of 250 mg/kg in rats with diabetes induced by streptozotocin (STZ) (Adisa et al., 2010). In addition to the hypoglycemic effect, they reduced lipid peroxidation and improved liver and renal function. The extract obtained from the leaves and branches of *Connarus lambertti* inhibited the microsomal glucose-6-phosphatase enzyme in an in vitro assay, indicating that this plant could be used as an adjuvant to control hyperglycemia in diabetic patients (Jiménez et al., 2001).

A hydroethanolic extract of *R. coccinea* leaves showed hypoglycemic effect on alloxan-induced diabetic rats at different dosages, which could be related to the presence of saponins in the extract (Dada et al., 2013). In addition to the reduction in glycemia, the oxidative stress markers improved for the 800 mg/kg dose, which indicates the great potential of this plant for future clinical applications in diabetes control. The hydroethanolic fraction of *R. cuspidata* extract demonstrated hypoglycemic activity at a dose of 200 mg/kg in rats with STZ-induced diabetes in a 28-day study (Laikowski et al., 2017). In *R. minor*, the ethanolic and aqueous extracts obtained from the roots promoted blood glucose reduction in rats with STZ-induced diabetes for both the 200 and 400 mg/kg dose in a 15-day exposure (Kulkarni et al., 2014). These authors attributed the hypoglycemic effect to the synergistic effect of the chemical compounds present in this plant extract.

3.3.11. Triglyceride and/or cholesterol reducing activity

Hydroethanolic extract of *R. coccinea* leaves exhibited cholesterol and triglyceride-lowering activity in rats after administration of 100, 200, and 400 mg/kg dosages for 56 weeks (Akindele et al., 2014). The authors attributed these effects to the presence of flavonoids and other phytoconstituents in the extract of this plant but did not chemically identify them (Akindele et al., 2014). In another study with the same species, the hydroethanolic extract of the leaves lowered total cholesterol and increased HDL fraction in diabetic rats (Dada et al., 2013).

For *R. minor*, ethanolic and aqueous extracts obtained from the barks reduced the total cholesterol and triglycerides, and increased serum HDL cholesterol level in diabetic rats at a dose of 400 mg in a 15-day exposure (Kulkarni et al., 2014).

3.3.12. Antibacterial activity

Methanolic extract obtained from *A. borneensis* bark and leaves was active in the disc diffusion assay against gram-positive bacteria *Staphylococcus aureus* (Chung et al., 2004). This study was conducted only from dry crude extract, and no studies have elucidated which compounds are responsible for this antibacterial action.

A study to evaluate the antimicrobial activity of *Cnestis ferruginea* fruits demonstrated activity against different bacterial strains, in which the aqueous extract of fruits presented MICs of 5–10 mg/mL against 11 different clinical isolates including *Streptococcus aureus, S. mutans, Escherichia coli, Citrobacter freundii,* among others (Ndukwe et al., 2005). In another investigation, different solvents were used to extract secondary metabolites which were submitted to antimicrobial evaluation (Kouakou et al., 2019). These authors demonstrated that aqueous extract had the best antimicrobial profile and was active against 10 of the 11 strains tested, including both gram-negative and positive bacteria, and this effect was attributed to the presence of hydroquine and caffeic acid methyl ester among the secondary metabolites identified in this plant (Kouakou et al., 2019). The antimicrobial effect of *Cnestis ferruginea* methanolic extract was proven against multidrug resistant (MDR) bacterial strains, by employing disk diffusion method against different MDR strains of *Streptococcus aureus*, *E. coli*, and *Salmonella* spp. impregnated with 7.5 mg/mL of the ethanolic extract of the stems, with similar inhibition to the erythromycin antibiotic (EC et al., 2015). For *Cnestis palala*, antibacterial activity was found in the ethanolic and crude extract fraction against gram-positive bacteria *Streptococcus aureus* and *Streptococcus epidermidis,* in different parts of the plant. The authors isolated the hydroquinone compound and related it to the antimicrobial effect of this plant species (Dej-adisai et al., 2015). Despite the promising result against tuberculosis-causing bacteria, no further studies have been conducted to clarify the chemical constitution and safety of using this plant.

The antimicrobial effect of aqueous extract of *Connarus favosus* bark revealed activity against *Morganella morganii* and *Serratia marcescens* inhibited at 250 μg/mL; *Yersinia enterocolitica* at 125.0 μg/mL; and *Pseudomonas fluorescens* at 15.6 μg/mL (Silva et al., 2016). Apparently, this effect is related to phenols and tannins present in the extract at high concentrations. *Connarus ruber* shoot extract was active against *Enterococcus faecalis* in the disk diffusion assay (Castilho et al., 2014). In addition to the antimicrobial effect, crude extract, n-butanolic, and aqueous fractions were significantly active against *E. faecalis* biofilm formation, and *Connarus ruber* could be used in dentistry against formation of biofilm associated with virulence of this microorganism (Castilho et al., 2013). In *R. coccinea,* antimicrobial activity was demonstrated for the n-butanolic and ethyl acetate fractions against *E. coli, Streptococcus aureus,* and *Salmonella typhi* bacteria; this activity is related to the presence of flavonoids and tannins, which were found in both fractions of the plant extract (Ahmadu et al., 2006). Different extracts obtained from the root bark of *R. obliquifoliolata* showed activity against strains of the genus *Vibrio, Shigella,* and *Campylobacter* at MICs 31.25, 62.5, and 125 μg/mL, respectively, which was attributed to the presence of tannins and other secondary metabolites in this plant (Longanga et al., 2000a); however, the chemical compounds responsible for this activity have not been identified yet. The extract obtained from *R. coccinea* leaves exhibited antibacterial activity against *Streptococcus aureus, E. coli,* and *Salmonella typhi* species with MICs of 1.75, 0.44, and 1.75 mg/mL, respectively (Ahmadu et al., 2006).

3.3.13. Antifungal activity

The ethanolic extract of *Cnestis palala* seeds showed similar activity to the ketoconazole pattern in disc diffusion assays against dermatophytes *Trichophyton mentagrophytes*, *T. rubrum,* and *Microsporum gyseum* (Dej-adisai et al., 2015). Additionally, the authors were able to demonstrate activity against *Candida albicans* yeast fungus for ethanolic

extracts of seeds, bark, and roots, and these effects were compared to the amphotericin B pattern (Dej-adisai et al., 2015). Antifungal activity was also demonstrated for n-butanolic extract obtained from *R. coccinea* leaves against *C. albicans* isolates with MIC of 0.88 mg/mL (Ahmadu et al., 2006).

3.3.14. Antiprotozoal activity

Different fractions of ethyl acetate extract obtained from *Connarus suberosus* barks exhibited activity against *Leishmania (Leishmania) amazonensis* with IC_{50} 27.57–94.00 μg/mL (Costa et al., 2014). Although the authors identified different chemical compounds in this plant extract, the chemical identity of the compounds responsible for the action against *L. amazonensis* could not be established. For *Connarus suberosus*, the hexane extract of root bark and wood demonstrated activity against *Trypanosma falciparum* and *T. brucei gambiense*, causative agents of malaria (Charneau et al., 2015). In this study, the extract was active against the free and intracellular forms of the *T. falciparum* protozoan. The erythrocyte forms had a the IC_{50} of 1.2 ± 0.5 μg/mL for the root wood extract and 1.2 ± 0.3 μg/mL for the root bark extract (Charneau et al., 2015). In the evaluation against the bloodstream forms of *T. brucei gambiense*, the IC_{50} was 1.7 ± 0.1 μg/mL for wood extract and 1.8 ± 0.0 μg/mL for the bark (Charneau et al., 2015). Methanolic extract from *M. expansa* barks exhibited actitation against *Trypanosma brucei brucei* at IC_{50} 37.0 ± 3.1 μg/mL; however, the authors did not continue investigating this plant because the species *T. falciparum* had an IC_{50}% higher than 64 μg/mL (Mesia et al., 2008). The dichloromethane fraction extracted from aerial parts of *R. coccinea* showed activity against *T. brucei brucei* IC_{50}% 14.7 ± 1.2 μg/mL; therefore, the potential of the plant species as an antiprotozoal agent indicates the need for isolation and identification of the substances responsible for the biological effect against the causative agent of African trypanosomiasis (Bero et al., 2009). The dichloromethane extract obtained from the aerial parts of *R. coccinea* had an IC_{50}% of 41.6 ± 22.1 μg/mL against *P. falciparum* (3D7) (Bero et al., 2009).

3.3.15. Antiviral activity

Connarus cochinchinensis showed antiviral activity involving the measles virus (Zarr, 2010). The methanolic extract of leaves administered concomitantly with the virus was able to decrease viral infection of Vero cells and was highly active against this pathogen. Those authors indicated that saponins, which are present among the secondary metabolites of this plant, may exert an antiviral effect.

3.3.16. Restoration of sexual function, fertility, and activity in gonads

In *Cnestis ferruginea*, the aqueous extract of the roots administered to rats at a dose of 52 mg/kg stimulated sexual activity by increasing testosterone levels in these animals (Toyin and Olaide, 2012). The aqueous extract from *R. coccinea* roots administered to rats with paroxetine-induced sexual dysfunction improved sexual performance and reestablished testicular function at a dose of 150 mg/kg, which may be related to increased testosterone and nitric oxide synthesis; however, the authors did not isolate and characterize the active principles responsible for the observed effects (Yakubu and Atoyebi, 2018).

The aqueous extract of *Cnestis ferruginea* leaves administered to female rats at a concentration of 50 and 100 mg/kg was able to promote hormonal changes responsible for increased fertility in these animals (Zougrou et al., 2016). In the 30-day study both doses were able to increase serum levels of the hormones FSH, LH, estradiol, progesterone, and prolactin. Evidences suggest that the presence of estrogenic compounds in the extract may be responsible for the effect observed in these animals (Zougrou et al., 2016).

Hydroethanolic extract of *R. coccinea* leaves administered at doses of 25 and 50 mg/kg to rats exhibited uterotonic effect (Amos et al., 2002). According to the authors, the effect of the extract may be antagonized by administration of the β-receptor agonist salbutamol, indicating that the uterotonic effect is mediated via the adrenergic pathway.

3.3.17. Antidiarrheal activity

For *R. coccinea* the aqueous extract obtained from the leaves had an antidiarrheal effect in mice treated with 400 mg/kg, which may be related to inhibition of α-adrenergic receptors that control intestinal propulsion and intestinal fluid secretion (Akindele and Adeyemi, 2006). In this study, different classes of chemical compounds were identified in the plant extract; however, the authors did not conduct experiments to elucidate which compounds are responsible for the effect. A methanolic extract of *R. obliquifoliolata* demonstrated antidiarrheal activity at the dose of 750 mg/kg in rats measured by decreasing the frequency of defecation and water loss through the stool (Longanga et al., 2001).

3.3.18. Snakebites

Aqueous extract from *Connarus favosus* roots proved to be effective in completely blocking lyophilized *Bothrops jararaca* venom-induced hemorrhage in mice (Silva et al., 2016). In this experiment, the antihemorrhagic effect was observed for the diluted venom extract at a concentration of 12/1 p/p, and according to the authors, the antiophidic effect is probably associated with the blockage of venom metalloproteinases by the phenolic compounds contained in the plant extract (Silva et al., 2016).

3.3.19. Genotoxicity suppression

The extract obtained from *Connarus ruber* bark added to water from mice exposed to cigarette smoke at a dose of 758 ppm was able to reduce the appearance of micronucleated reticulocytes and erythrocytes in these animals (Nakamura et al., 2011).

3.3.20. Antiproliferative activity

The aqueous extract of *Connarus angustifolius* leaves was moderately active against human colon carcinoma cells (KM-12), with a lethality of 15% for the tested extract at a concentration of 100 μg/mL (Suffredini et al., 2007). This study reflects the need for fractionation of the extract to identify more active fractions, as well as to isolate and identify which compounds are responsible for the antiproliferative effect demonstrated in the in vitro study. Methanolic extract from *M. expansa* bark demonstrated cytotoxic activity against the human lung fibroblast strain (MRC-5) IC_{50}% 27.0 ± 2.2 μg/mL (Mesia et al., 2008). The organic extract (dichloromethane/methanol 1/1) from *P. macrophyllus* aerial parts was moderately active against human colon carcinoma cells (KM-12) with a lethality of 15% for the tested extract at 100 μg/ml (Suffredini et al., 2007). Different fractions of the ethanolic/ethanolic extract (1:1) of *R. coccinea* leaves showed antiproliferative activity for HT29 cells at concentrations of 100 μg/mL (Akindele et al., 2016, 2011). However, no experiments were conducted to elucidate the chemical constituents responsible for this effect, although the authors stated that the antiproliferative effect of this plant is due to the synergistic effect of different constituents present in the extract (Akindele et al., 2011). Another study using the same plant species identified an antiproliferative effect of methanolic extract at a concentration of 200 μg/mL against human cells derived from breast (BT-549, BT-20) and prostate (PC-3) adenocarcinomas (Fadeyi et al., 2013). According to the authors, extracts of this plant can be considered very active as some cell lines inhibited ≥50% in cells treated with the extract at a concentration of 20 μg/mL. The authors finally emphasized the need for bioguided studies to elucidate which chemical compounds are responsible for the antiproliferative action of plant extracts.

3.4. Toxicological

The potentially toxic substances has been reported for some species of Connaraceae (Garon et al., 2007; Jeannoda et al., 1985; Vickery and Vickery, 1974), although only a few species of the family have been investigated. Therefore, the safety remains unknown for most species

reported in ethnobotanical and/or pharmacological studies.

The presence of certain coumarin derived compounds may be related to anticoagulant effects responsible for rabbit death (Vickery and Vickery, 1980). A study on the toxicity to rabbits of the leaves of different Connaraceae species demonstrated different symptoms in vivo and postmortem evaluation of the animals. The LD_{50} of *A. pentagyna* leaves was established at a dose between 20 and 50 g/kg, and the main effects observed were the onset of convulsions, hyperhidrosis, conjunctivitis, liver alterations, and tracheal edema, with death occurring between 2 and 5 days after rabbits exposure (Vickery and Vickery, 1974). For the species *Cnestis corniculata, Cnestis ferruginea,* and *R. coccinea*, LD_{50}s were 10, 25, and 50 g/kg, respectively, and the toxic effects observed in rabbits were similar to those found for *A. pentagyna* (Vickery and Vickery, 1974).

The compound L-methionine sulfoximine (MSX) has neurotoxic action and was found in the extract obtained from *Cnestis ferruginea* leaves (Garon et al., 2007) and in different parts of *Cnestis palala* (Murakoshi et al., 1993). For *Cnestis ferruginea*, the presence of MSX was demonstrated for the aqueous extract of the plant in a study that measured cell viability (Chinese Hamster Ovary (CHOK1) cells) by (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)- 5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide) (XTT) and found viability reduced as the extract concentration increased (Garon et al., 2007). Considering the toxic effects of MSX, the decrease in viability could be related to the presence of that compound. In *Cnestis palala*, the toxicological effect of ground seeds was evaluated at a dose of 347 mg/kg in dogs, and the observed effects included the onset of vomiting in the first hours after exposure, locomotor and respiratory disorders, salivation, and seizures ultimately leading to death 24 h after exposure (Murakoshi et al., 1993). The toxic effects observed in these two species are directly related to the MSX compound. Therefore, the presence of MSX in extracts of Connaraceae attributed to human use may have negative impact on safety profile of the species. The presence of such compounds should be discarded while preparing samples of Connaraceae for pharmacological studies or producing medicines from these plant sources.

A study with the fraction of alkaloids obtained from *Cnestis ferruginea* roots observed enzymatic and histological changes (Atere and Ajao, 2009). In this study, the administration of alkaloids rich extract at doses of 3, 6, and 9 mg/kg increased the serum concentration of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) and promoted changes in liver tissue histology in the largest doses tested (Atere and Ajao, 2009). However, the popular uses of this plant do not use the enriched fractions of the extract, but only the preparation of decocts and macerates, and thus the findings of that study cannot be extrapolated to the hepatotoxicity forms of the traditional preparation. Moreover, recent studies with *Cnestis ferruginea* have indicated a toxicological profile favorable to future pharmacological applications of this plant (Hong and Lyu, 2011; Ishola et al., 2011). The acute toxicity of methanolic extract obtained from *Cnestis ferruginea* roots was assessed by oral administration to rats at doses of 1, 2, 4, 8, and 10 g/kg (Ishola et al., 2011). At doses of 1 and 2 g/kg no deaths occurred; however, at the dose of 10 g/kg, 100% of the experimental animals died within 24 h of administration (Ishola et al., 2011). The authors were able to establish that the LD_{50} of the oral dry methanolic extract for this plant is 5.22 g/kg and that oral administration of this plant can be considered safe as $LD_{50} \geq 5.00$ g/kg. Another study on sub-chronic toxicity of methanolic extract from *Cnestis ferruginea* roots demonstrated the relative safety of this plant, which can also induce antioxidant enzymes in rats (Ishola et al., 2012b). These authors elucidated the effect of the extract at different dosages against biochemical, histological, and hematological parameters, and the main effects were related to the absence of sperm production at a concentration of 1,000 mg/kg and reduced body weight with some tested dosages, although most of the deleterious effects were in much higher dosages than those used by traditional medicine. The genotoxicity of *Cnestis ferruginea* was ruled out for whole plant methanolic extract in a mutagenicity assay with *Salmonella typhimurium* and *E. coli* WPuvr (Hong and Lyu, 2011).

In the genus *Connarus*, the evaluation of *Connarus cochinchinensis* in the toxicological assay of methanolic extract of leaves at concentrations of 10 to 1,000 ppm against *Artemia salina* had an LD_{50} of over 1,000 mg/L, demonstrating a favorable safety profile of this species (Zarr, 2010). In the cytotoxicity assay with Vero Cells, the extract of this plant presented toxicity with cellular cytotoxicity value (CTC_{50}) at 104.5 mg/L (Zarr, 2010). Preliminary toxicity evaluation of the ethanolic extract of *Connarus lambertii* leaves and twigs in an assay with *A. salina* revealed that this species has microcrustacean toxicity, and the LD_{50} of the extract for these animals was established at 0.15 mg/mL (Jiménez et al., 2001). In the same species, aqueous extract of the leaves presented an LD_{50} of 16.52 mg/mL against *A. salina* (Coe et al., 2010). The difference in the solvents used may be the reason for the discrepancies of the values found in these two studies. Administered orally to mice at doses between 500 and 2,000 mg/kg, the aqueous extract of *Connarus ruber* barks did not cause mortality (Nakamura et al., 2011). The toxicological activity of the water-soluble extract and the ethanolic fraction of *Connarus suberosus* barks was evaluated after the mice were exposed to different doses of intraperitoneally administered extracts, and the water soluble fraction presented a LD_{50} of 310 ± 52 mg/kg, while the ethanolic fraction was 210 ± 22 mg/kg (Taveira et al., 1988). According to these authors, *Connarus suberosus* has toxicological potential as the LD_{50} of the extracts are less than 500 mg/kg. As this species is used for traditional medicinal purposes (Costa et al., 2014; Taveira et al., 1988), other experiments should be conducted to confirm the safety of using this plant.

The acute toxicity of hydroethanolic extract obtained from *R. coccinea* leaves was assessed by oral and intraperitoneal routes in rats (Akindele et al., 2014). Orally administered at a dose of 10 g/kg for 14 days, the extract did not kill the animals; however, intraperitoneal administration had a 100% mortality rate at 800 mg/kg, and the LD_{50} for this extract was set at 288.40 mg/kg (Akindele et al., 2014) The toxicological potential is similar to that found for *Connarus suberosus* and demands additional studies to evaluate its toxicity. An evaluation of the acute toxicological potential of ethanolic extract of *R. coccinea* barks indicated a favorable safety profile for the use of this plant (Kossivi et al., 2015). In this study administered orally at the dose of 5,000 mg/kg to rats, no behavioral changes and mortality were observed. At sub-chronic exposure to the same extract for 28 days, no enzymatic, hematologic, and vital organ changes were observed in the animals (Kossivi et al., 2015).

The acute toxicity of aqueous extract of *R. coccinea* leaves was evaluated by oral and intraperitoneal routes in mice (Akindele and Adeyemi, 2006). When orally administered at a dose of 10 g/kg, the extract did not produce mortality, whereas for intraperitoneal administration, the LD_{50} was set at 141.3 mg/kg. In another acute exposure study of the same species in rats, intraperitoneally administered hydroethanolic leaf extract had an LD_{50} of 547.7 mg/kg (Amos et al., 2002). The differences found between these studies for the LD_{50} may be related to the different extract types and breeds of rats used in the experiments. Studies with ethanolic extract of *R. induta* leaves against *A. salina* assay and in tube plaque hemolysis tests at concentrations of 1,000–10 μg/mL presented a favorable safety profile for the use of this plant (Kalegari et al., 2011). That study demonstrated that even at the highest dose tested, the extract did not cause mortality of *A. salina* or promote hemolysis in either assay. A study evaluating acute oral toxicity of aqueous and ethanolic extracts of *R. minor* roots in rats at different doses between 10 and 3,000 mg/kg demonstrated a safety profile favorable to the medicinal use of this species, since no mortality was observed at the doses tested (Kulkarni et al., 2014).

3.5. Development of research for the species

This pioneering review compiled and analyzed data to present a

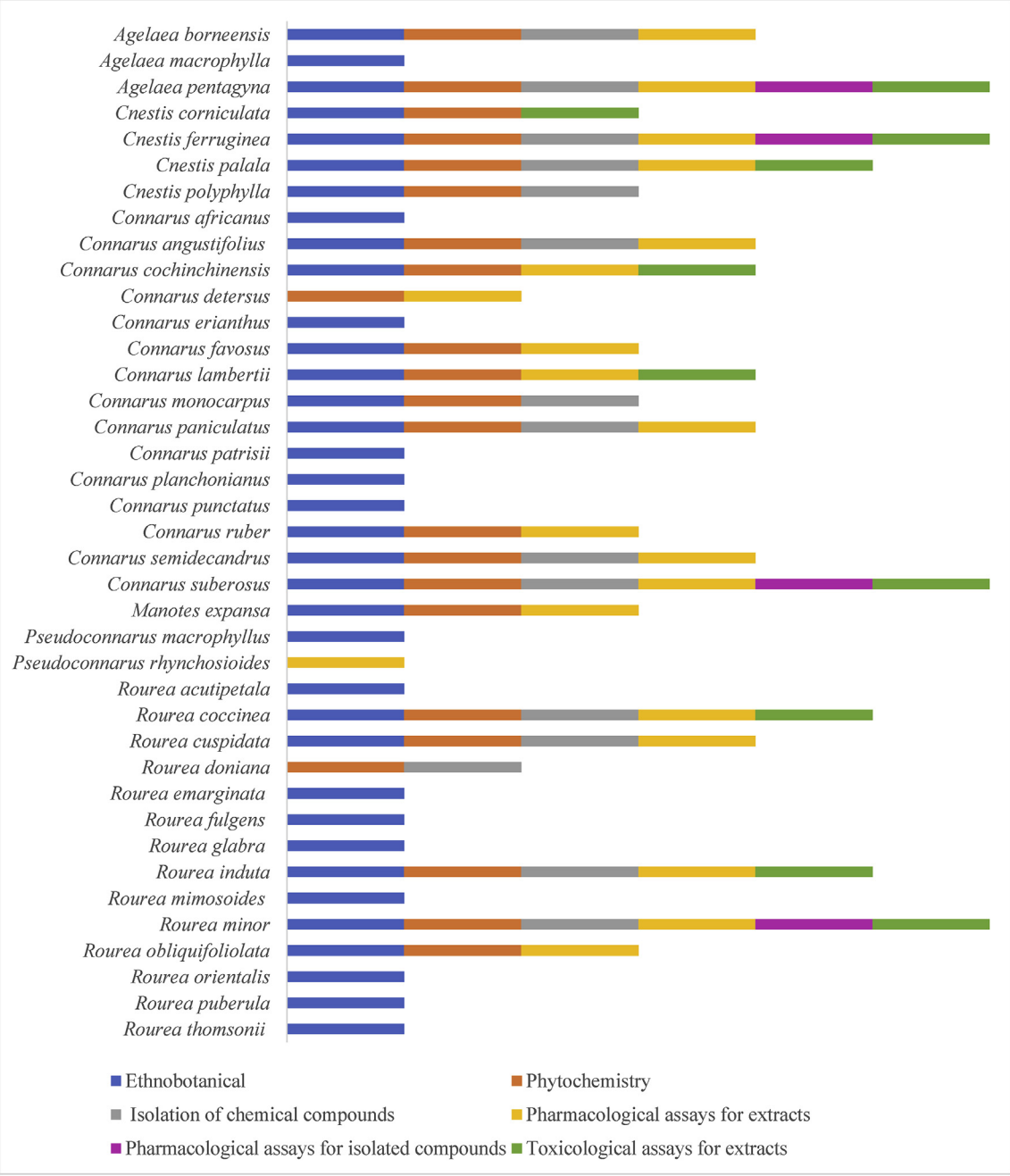

Fig. 6. Overview of research with different Connaraceaes species.

general overview of research about the pharmacological potential in Connaraceae, and summarized in which type of study each species was analyzed (Fig. 6). The degree of research development for each species included in the present study was inferred based on the available information in the literature for the following criteria: ethnobotanical, phytochemistry, isolation of chemical compounds, pharmacological assays of extracts, pharmacological assays about isolated compounds, and toxicological assays of extracts. This indicative measurement was applied to each species that were studied according to these approaches. Our results elucidate that *Agelaea pentagyna, Cnestis ferruginea, Connars suberosus,* and *Rourea minor* present the highest research development value. This indicates that compounds from these four species are the best candidates to produce medicines for human use, although additional experiments are still required. In addition, clinical assays using Connaraceae have never been reported in the literature, evidencing a knowledge-gap that should be thoroughly investigated.

4. Conclusions and future perspectives

In this review, 39 species of Connaraceaes were found with associated pharmacological potential. Considering the recognized total of taxa (200) for this plant family, approximately 80% of the species have not been studied by any published work to elucidate their chemical and pharmacological potential. Among the 39 species reported here with associated pharmacological potential, 24 are unknown in terms of chemical constitution, which includes all representatives of *Manotes* and *Pseudoconnarus*. Medicinal studies using the species of these genera should be encouraged before their populations are destroyed, considering that they inhabit tropical forests which are constantly anthropized by deforestation, burning, urbanization, and expansion of

agricultural frontiers. Such studies could support public policies for the preservation and sustainable use of these pharmacological resources, also important for the conservation of the ecosystems they occur.

Several Connaraceae species are widely used as a source of traditional medicines around the world. Nevertheless, studies to identify biological activities for chemical compounds isolated from these species are still rare. Further research with these species must be conducted to scientifically confirm the pharmacological activities reported by popular use, which contributes to the discovery of new therapeutic alternatives, as well as to stimulate protection measures of natural areas.

Comparing the activities reported by traditional use with the pharmacological activities described in this review elucidates that many remedies have not been studied but should be investigated. The use of some Connaraceae species in ethnobotany, such as *Rourea induta (*to treat of Chagas disease), *Connarus cochinchinensis* (to treat tuberculose), and *Agelaea. pentagyna*, *Cnestis palala*, *Cnestis polyphylla*, *Connarus* angustifolius and *Pseudoconnarus* rhynchosioides (to treat malaria), are examples of species in which secondary compounds may lead to new medications for the treatment of these important human pathologies. Therefore, this plant family deserves special attention of the scientific community for future research on human medicine.

Studies with a toxicological approach cover only 10 (25%) of the 39 Connaraceaes species compiled here. Therefore, it is important that additional species of the family are investigated to evaluate if they are safe for use in human medicine, as several taxa have been widely used in popular medicine. The available data indicate that few Connaraceae compounds present toxicological risks to human health; nevertheless, the presence of such substances must be ruled out before it is used as a medicinal source.

Finally, the systematized data demonstrate that the Connaraceae family has enormous potential to contribute to the discovery of new therapeutic options, especially the species *Agelaea pentagyna, Cnestis ferruginea, Connarus suberosus,* and *Rourea minor*, which are the most commonly studied in pharmacological experiments of isolated compounds. For these species, clinical studies are needed to scientifically prove their effectiveness and safety prior to production of medicines for human consumption.

Authors' contributions

Luis Fernando N. A. Paim collected the information and wrote the manuscript; Cássio A. P. Toledo, Joicelene R. L. da Paz, Aline Picolotto, and Guilhereme Ballardin assisted in the modification and adaptation of the text. Sidnei Moura and Mirian Salvador made the final revision of the manuscript. All authors approved the final submitted version of the manuscript.

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Appendix A. Supplementary data

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Capítulo 2

Four almost unexplored species of Brazilian Connarus (Connaraceae): Chemical composition by ESI-QTof-MS/MS–GNPS and a pharmacologic potential

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RESEARCH ARTICLE

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Four almost unexplored species of Brazilian *Connarus* (Connaraceae): Chemical composition by ESI-QTof-MS/MS–GNPS and a pharmacologic potential

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Abstract

Introduction: Species of Connaraceae are globally used in traditional medicines. However, several of these have not been studied regarding their chemical composition, and some are even at risk of extinction without proper studies. Therefore, the chemical composition and pharmacological potential of *Connarus blanchetii* Planch., *Connarus nodosus* Baker, *Connarus regnellii* G. Schellenb., and *Connarus suberosus* Planch., which were previously unknown, were analyzed.

Objective: This work aims to investigate the pharmacological potential of these four *Connarus* species. The chemical composition of different extracts was determined by high-resolution mass spectrometry (HRMS), with subsequent analysis by the GNPS platform and competitive fragmentation modeling (CFM).

Materials and methods: Leaf extracts (*C. blanchetii*, *C. nodosus*, *C. regnellii*, and *C. suberosus*) and bark extracts (*C. regnellii* and *C. suberosus*) were obtained by decoction, infusion, and maceration. LC/HRMS data were submitted to the GNPS platform and evaluated using CFM in order to confirm the structures.

Results: The HRMS–GNPS/CFM analysis indicated the presence of 23 compounds that were mainly identified as phenolic derivatives from quercetin and myricetin, of which 21 are unedited in the *Connarus* genus. Thus, from the analyses performed, we can identify different compounds with pharmacological potential, as well as the most suitable forms of extraction.

Conclusion: Using HRMS–GNPS/CFM, 21 unpublished compounds were identified in the studied species. Therefore, our combination of data analysis techniques can be used to determine their chemical composition.

KEYWORDS
Connaraceae, extractive methods, high-resolution mass spectrometry, molecular network, phenolic compounds

1 | INTRODUCTION

The family Connaraceae is represented by 12–13 genera and about 200 species, mostly distributed in tropical forests and savannas from South America, Central Africa, and Southeast Asia.[1] In Brazil, five genera of Connaraceae are found (*Bernardinia*, *Cnestidium*, *Connarus*, *Pseudoconnarus*, and *Rourea* – if the former is considered separate from *Rourea*), adding up to 75 species, most of which are restricted to the Brazilian territory.[2]

Species of this family have been used around the world in traditional medicines with diverse applications, namely to the treatment of inflammation, fever, pain, hypertension, and diabetes.[3–7] Some of the Brazilian taxa have been highlighted, including *Connarus favosus* Planch., which is popularly used as an antiophidic,[8] *Connarus angustifolius* (Radlk.) G. Schellenb. for the treatment of inflammation,[9] *Connarus suberosus* Planch. for heart disease,[10] *Rourea cuspidata* Benth. ex Baker for diabetes,[11] and *Rourea induta* Planch., traditionally used in rheumatic and Chagas diseases.[12]

The bioactivities of these species are directly associated with the identified chemical composition, with phenolic being the most prominent compound[11–13]: Kalegari et al. (2014) demonstrated that the main compound in *R. induta* is hyperin, while Pires et al. (2017) demonstrated the presence of rutin and quercetrin in *C. angustifolius*. However, as noted in a previous review,[14] among 39 potentially medicinal species of Connaraceae, only 10 have had their chemical composition specified, with only a few demonstrative studies.

In addition, reported extract preparations have been quite heterogeneous, both in scientific work and in traditional medicine. Examples include decoction,[15–17] infusion,[18] and maceration,[19] which may lead to the extraction of different compounds. Some authors have already demonstrated that there are changes in the quantitative profile of metabolites for different plant species when comparing the different extractive methods tested.[20,21]

The present work aims to identify new chemical compounds from four *Connarus* species and to qualitatively evaluate possible changes in terms of the main methods used by traditional medicine. We intend to analyze the chemical composition of four species from the genus *Connarus* – the largest of the family – and consequently expand the knowledge on the pharmacological potential in Connaraceae. High-resolution mass spectrometry (HRMS) has been highlighted for the analysis of natural compounds in complex mixtures in plant extracts, such as phenolics in *Rourea*.[11] Databases have recently been created based on the exact *m/z*, isotopic ratio, and fragmentation pathway, to assist in confirming the metabolic profile. The Global Natural Products Social Molecular Networking (GNPS) platform allows acceleration of the discovery of new bioactive compounds,[22,23] providing valuable information of new natural products to the scientific community, to be used as spectral reference libraries.[24] Moreover, we propose to use competitive fragmentation modeling (CFM) in order to confirm the identity of the compounds.

This work aims to evaluate the chemical composition of four species (*Connarus blanchetii* Planch., *C. nodosus* Baker, *C. regnellii* G. Schellenb., and *C. suberosus* Planch.), present in different Brazilian biomes. For this, different plant parts were submitted to different extractive methods (decoction, infusion, and maceration). Thus, we can evaluate the pharmacological potential from this work, supporting future research emphasizing the potential of Brazil's biodiversity. Experimentally, the chemical composition of different extracts was established by HRMS with use of the GNPS platform and CFM.

2 | EXPERIMENTAL PROCEDURES

2.1 | Materials

Ethanol, methanol and formic acid analytical grade were obtained from Merck (Barueri, SP, Brazil). Ultra-pure water was obtained using a Milli-Q Plus® system (Millipore, Bedford, USA). Dihydroquercetin, kaempferol, quercetin, and rutin were purchased from Sigma-Aldrich Brazil Ltda (São Paulo, Brazil).

2.2 | Plant species

Plant accessions of *Connarus blanchetii*, *C. nodosus*, *C. regnellii*, and *C. suberosus* were registered at the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) and additional information on these species is presented in Table 1. They were individually dehydrated in a greenhouse

TABLE 1 Plant material of Brazilian species of *Connarus* (Connaraceae) used in this work

Species	Acronyms[a]	Location	Biome	Coordinates	Voucher (herbarium)	SisGen
C. blanchetii	CBL	Ilhéus, BA.	Atlantic Forest	14°55′56″S, 39°1′32″W	C. Toledo (ESA** 143609)	A1FE9E7
C. nodosus	CBL	Rio de Janeiro, RJ.	Atlantic Forest	23°24′67″S, 43°32′07″W	C. Toledo (ESA 143625)	AD26B71
C. regnellii	CRB CRL	Piedade, SP.	Atlantic Forest	23°42'05.5"S, 47°30'38.5"W	C. Toledo (ESA 143607)	A7E69F6
C. suberosus	CSB CSL	Brasília, DF.	Cerrado	16°56.54″S, 47°51.58′W	J. Paz (UB*** Paz 86)	A251027

[a]CBL, *C. blanchetii* leaves; CNL, *C. nodosus* leaves; CRB, *C. regnelli* bark; CRL, *C. regnelli* leaves; CSB, *C. suberosus* bark; CSL, *C. suberosus* leaves. **ESA, Escola Superior de Agricultura. ***UB, Universidade de Brasilia.

with dry air flow at a controlled temperature of 35°C for 7 days and, subsequently, they were ground in a knife mill, *Willye* Model TE 650 Tecnal®.

2.3 | Extraction methods

2.3.1 | Decoction (D)

Decoction was conducted following Oliveira et al. (2016),[25] with some modifications: 20 g of the obtained powder was added to water preheated at 100°C (100 mL) and maintained at a constant temperature of 100°C for 30 min under continual agitation. This procedure was repeated twice, the combined supernatants were decanted, centrifuged for 5 min at 3000 ×g and 20°C), and filtered (12–25 μm), the solvent was removed by rotative evaporation (Rotavapor® Buchi R210), and samples were lyophilized for 24 h in a freeze drier (Labconco Freezone® 4.5 Plus).

2.3.2 | Infusion (I)

Infusion was conducted following Kalegari et al. (2014),[12] with some modifications: 20 g of the obtained powder was infused with water preheated at 70°C (100 mL) for 30 min under continual agitation at room temperature. This procedure was repeated twice, the combined supernatants were decanted, centrifuged for 5 min at 3000 ×g and 20°C, and filtered (12–25 μm), the solvent was removed by rotative evaporation, and samples were lyophilized in a freeze drier.

2.3.3 | Maceration (tincture) (M)

This method was conducted according to Ayouni et al. (2016),[26] with some modifications: 20 g of the obtained powder was macerated twice in ethanol (100 mL) for 24 h under continual agitation at room temperature. After decantation and centrifugation for 5 min at 3000 ×*g* and 20°C), the recovered and combined supernatants were filtered (12–25 μm) and then solvent was removed by rotative evaporation.

2.4 | LC–MS analysis

LC–MS analysis was performed by a Shimadzu 20A HPLC system with binary solvent delivery, a degas system, an autosampler, and an SPD-20A UV–Visible detector (dual channel λ 254 and 320 nm). Separation was performed with an ODS C_{18} analytical column (4.6 × 250 mm), with particles of 5 μm. The mobile phase was in gradient mode (A, water/formic acid 0.1% v/v; B, methanol/formic acid 0.1% v/v), which were eluted at 1 mL min^{-1} as follows: 13.8% of B at 0–45 min; 28% of B at 45–60 min; 100% of B at 60–80 min, and finally 13.8% of B at 80–82 min. MS analysis was performed using a Bruker® MicroTof-QII spectrometer with electrospray ionization (ESI) source operated in positive ionization mode. The ESI source was operated at 200°C with an ionization voltage of 35–40 eV and a sheath gas flow rate of 8 L min^{-1}. The analysis was performed at an *m/z* range of 100–1,200 and a normalized collision energy of 10 eV at 15,000 resolution (FWHM) was used for the survey scans.

2.5 | Data analysis

A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu).[24] The spectra were window filtered by choosing only the top 6 fragment ions in the +/− 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da, with an MS/MS fragment ion tolerance of 0.02 Da.[27] A network was then created in which edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. In addition, edges between two nodes were only kept in the network if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS spectral libraries. The library spectra were filtered in the same manner as the input data. All matches between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks. The results were downloaded and exported to be visualized on Cytoscape 3.8.0 software [https://cytoscape.org].

2.6 | Competitive fragmentation modelling (CFM) – spectral prediction

To confirm the identification by the GNPS platform, in addition to the retention times of the metabolites present in the extracts, a spectral prediction tool was applied (https://cfmid.wishartlab.com). This tool provides low-energy (10 V), medium-energy (20 V), and high-energy (40 V) MS/MS spectra for a necessary input structure in the Simplified Molecular Input Line Entry System (SMILES) format. Spectra of compounds were produced using combinatorial fragmentation.[28,29] The SMILES of the compounds were obtained from the website (https://pubchem.ncbi.nlm.nih.gov/), and the data were then submitted to the workflow tool with the following parameters: spectrum type, ESI; ion mode, positive; adduct type, $[M + H]^+$. Spectral peaks and possible matching fragments for the compounds were evaluated with 40 V, a similar energy to that used in the LC/MS analysis.

3 | RESULTS AND DISCUSSION

Here, we submitted four species of *Connarus* to the extractive processes of decoction, infusion, and maceration, the most used methods

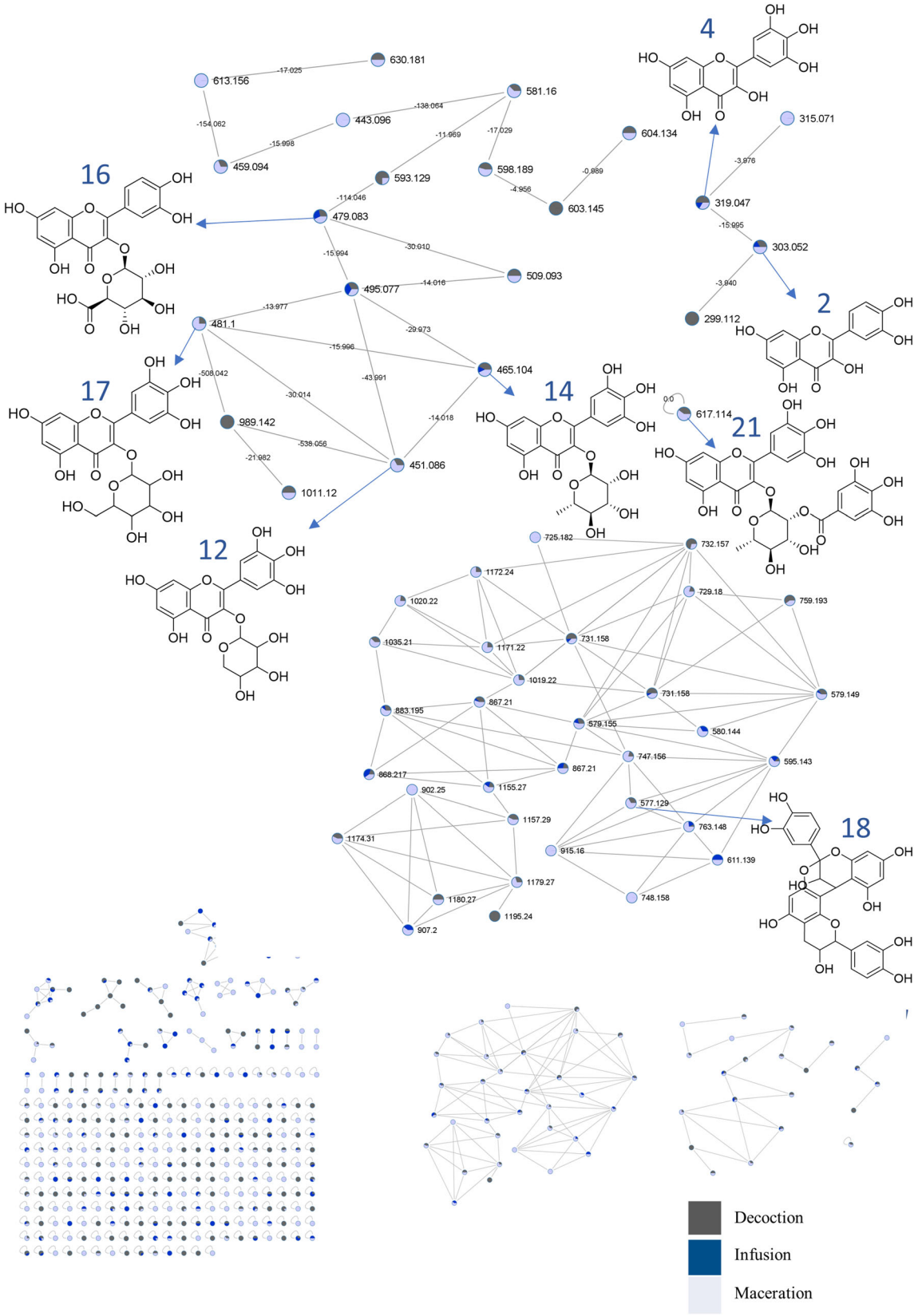

FIGURE 1 Clusters corresponding to compounds of *Connarus blanchetii* (Connaraceae) – Leaf extract with GNPS [Colour figure can be viewed at wileyonlinelibrary.com]

in traditional medicine and previously applied in species of Connaraceae.[13,15,19,30,31] Differences in the quantitative profile of secondary metabolites are described with different extractive processes for several plant species.[20,25] In a recent review, researchers found that only 10 out of 200 known Connaraceae species have their chemical composition established and, among the

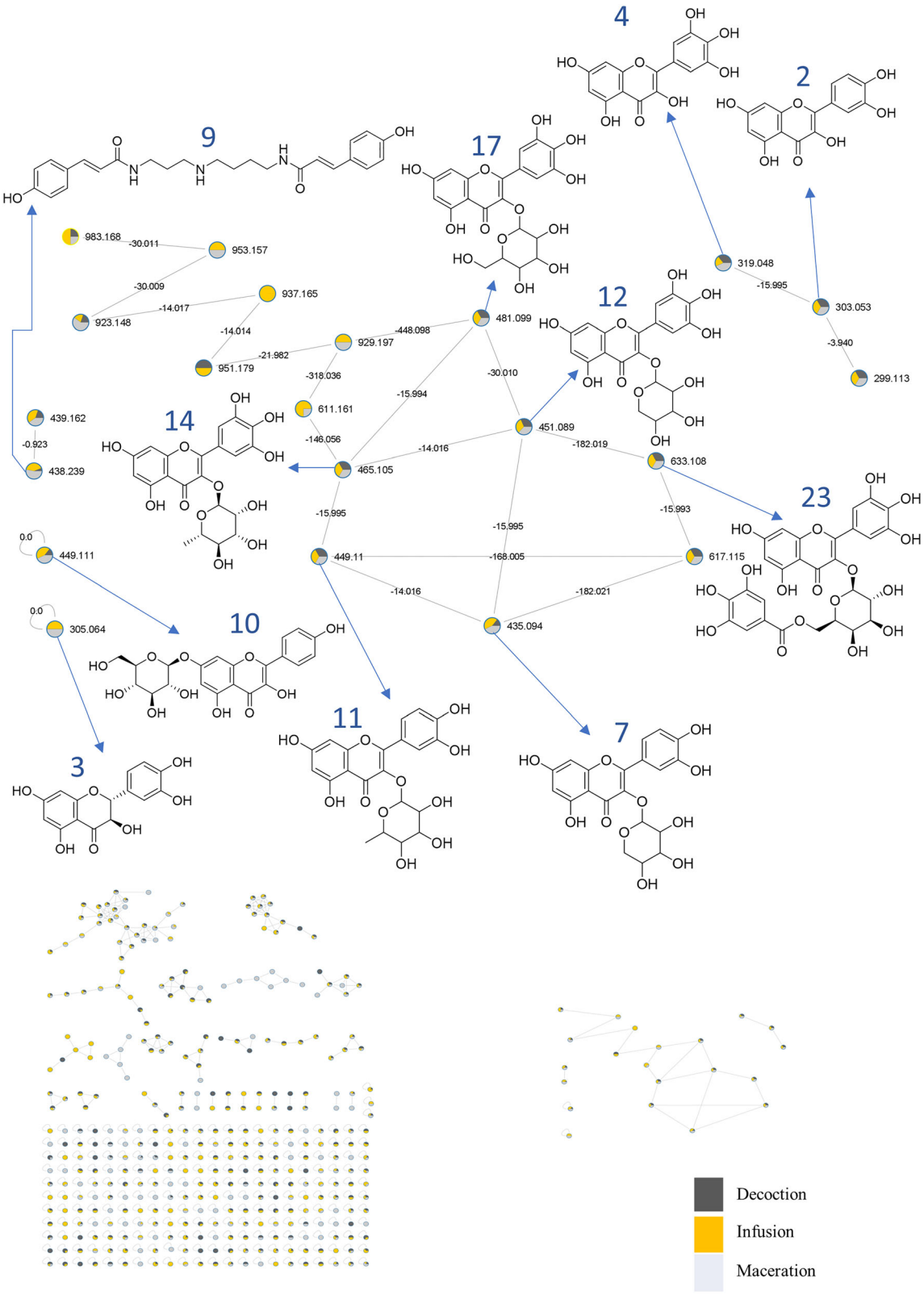

FIGURE 2 Clusters corresponding to compounds of *Connarus nodosus* (Connaraceae) – Leaf extract with GNPS [Colour figure can be viewed at wileyonlinelibrary.com]

genus *Connarus*, only a few chemical compounds have been identified at present. In Brazil, several Connaraceae are found in the Atlantic Forest and Cerrado biomes,[32,33] considering diversity hotspots[34–36] that harbor many species at imminent risk of extinction.[37–39]

Connaraceae includes plants of various life forms, including small shrubs, lianas, and trees.[1] With regard to the studied plants, both *C. blanchetii* and *C. nodosus* are shrubs or scandent shrubs, while *C. regnellii* and *C. suberosus* are trees; thus, only the leaves of the first two species were analyzed, with barks also evaluated for the latter two species. Among these four species, *C. suberosus* is traditionally known as "tropeiro"[10] and has been reported in folk medicine.[10,14,40] However, for *C. blanchetii*, *C. nodosus*, and *C. regnellii*, no ethnopharmacological report or study of chemical composition has been reported to date.

Different studies have shown that flavonoids and other phenolic compounds are amongst the main compounds found in Connaraceae.[3,7,11–13] Heterosides derived from quercetin have already been found in *Connarus* and *Rourea* and are implicated in most of the biological activities reported for the species already studied.[5–7,11,12,30]

Connarus blanchetii is shrubby, particularly found in the coastal Atlantic Forest of northeast Brazil.[2] We found that this species is not reported in the list of plants contemplated for ethnobotanical use and there is no study to date establishing its chemical composition.[14] In extracts of *C. blanchetii* leaves (CBL), the molecular network based on the similarity of MS/MS spectra generated on the GNPS platform identified the presence of 395 precursor ions visualized as nodes in the molecular network (Figure 1), including 31 clusters (node ≥ 2) and 364 unique nodes. From these nodes, eight compounds could be identified in the different CBL extracts. The aglycones quercetin and myricetin and the heterosides myricetin-3-*O*-pentoside, myricitrin, quercetin-3-glucuronide, myricetin-3-*O*-hexoside, and gallomyricitrin, together with proanthocyanidin A1, were the compounds identified in *C. blanchetii*.

In CBL, the largest number of nodes (100) appeared in the maceration method, followed by the decoction method (with 99 others). Among the eight compounds identified for CBL, four appeared among the three methods tested, while the others were measured only in decoction and maceration.

Four heterosides identified in CBL are derivatives from flavonoid myricetin. In the molecular network, the compounds myricetin-3-*O*-pentoside and myricitrin appeared with a mass unit variation (Δmu) of 14 Daltons (Da) in the mass unit, referring to a (CH_2) group of the sugar portion of the molecule, while quercetin (pentahydroxyflavone) and myricetin (hexahydroxyflavone) presented a Δmu of 16 Da for the hydroxylation of ring B of mircetin.

Connarus nodosus includes both shrubs and lianas of the Atlantic Forest, occurring from the state of Rio de Janeiro to the southern parts of the state of Bahia.[2] Like CBL, this species has no reports of ethnobotanical use or studies elucidating the chemical composition. For the leaves of *C. nodosus* (CNL), the molecular network identified the presence of 402 precursor ions visualized as nodes in the molecular map (Figure 2), including 30 clusters (node ≥ 2) and 372 unique nodes. Among these nodes, 11 compounds could be identified, including the aglycones quercetin, dihydroquercetin, and myricetin.

In CNL, 131 nodes could be identified among the three tested methods, of which 10 of the 11 compounds measured for the species were present. Only dihydroquercetin showed a different result and was identified in the infusion and maceration only. Therefore, the type of method chosen for CNL has minimal effects on the qualitative profile of the identified compounds.

In CNL, four heterosides are derived from myricetin and two others from quercetin. For myricetin derived compounds, myricetin 3-*O*-beta-D-galactoside 6″-*O*-gallate has a Δmu of 182 Da compared to myricetin-3-*O*-pentoside, representing the gallate group linked to the sugar portion of the molecule found in the first molecule. On the other hand, myricetin-3-*O*-hexoside presents a variation of 15 Da in relation to myricitrin, which is related to a hydroxylation of the sugar portion of the molecule. For quercetin derivatives, quercetin-3-*O*-pentoside and quercetin 3-rhamnoside present 14 Da variations, which refers to the group (CH_2) attached to the sugar portion of the molecule.

Connarus regnellii is a tree species from the Atlantic Forest of southeast Brazil.[2] This species is not reported for its traditional use and has no study of its chemical composition. For the bark of *C. regnellii* (CRB), the molecular network based on the similarity of MS/MS spectra generated on the GNPS identified the presence of 373 precursor ions visualized as nodes in the molecular map (Figure 3), including 31 clusters (node ≥ 2) and 342 unique nodes. In this analysis, four chemical compounds were identified from CRB, among them the aglycones quercetin and myricetin, the heteroside myricitrin, and proanthocyanidin A1.

In CRB, 133 nodes appeared among the three tested methods, of which all compounds identified for the species could be measured. The bark of *C. regnellii* presented an extensive cluster referring to the presence of procyanidins, where a high number of compounds can be seen among them, but only proanthocyanidin A1 could be identified. It seems that the bark of this species is qualitatively richer in anthocyanidin derivatives than it is in flavonoids.

In the leaves of *C. regnellii* (CRL), the GNPS platform identified the presence of 412 precursor ions visualized as nodes in the molecular map (Figure 4), including 35 clusters (node ≥ 2) and 377 unique nodes. In this species, eight compounds were identified, among them proanthocyanidin A1, kaempferol, quercetin, and five heterosides derived from quercetin.

In CRL the greatest number of nodes (142) was found using the three methods, which allowed the identification of all compounds seen in this species. Thus, comparing the identified compounds in CRL, the choice of method did not affect the qualitative profile of the metabolites. In CRL, in the cluster referring to the heterosides the derivatives of quercetin: quercetin-3-*O*-pentoside and quercetin 3-*O*-*rhamnoside* has a variation of 14 Da, which relates to the CH_2 group linked to the sugar portion of the latter compound.

Connarus suberosus is a tree widespread in the Brazilian Cerrado.[2] This species is used in traditional Brazilian medicine[10,40] and has some chemical compounds established, among them neoflavonoid,

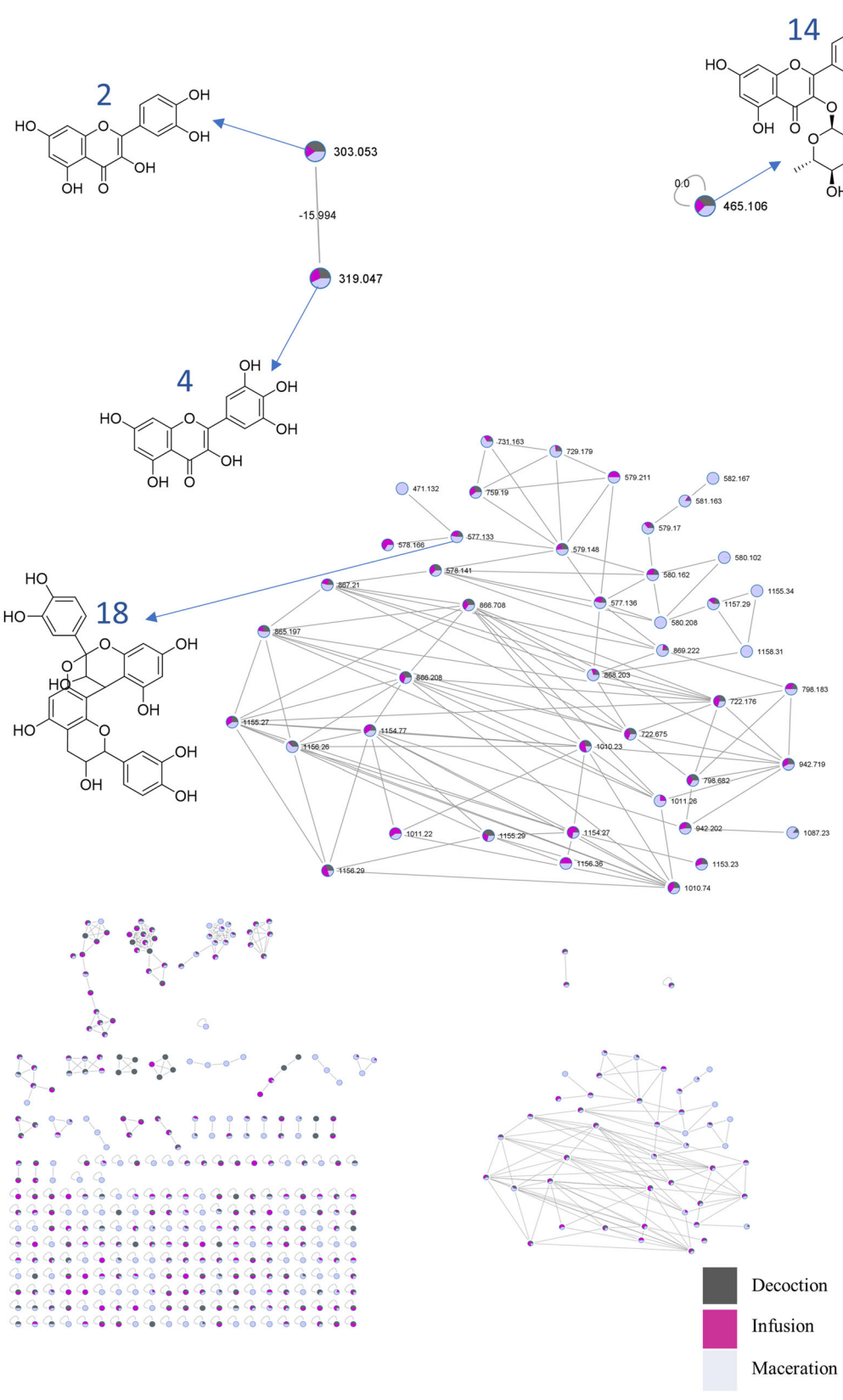

FIGURE 3 Clusters corresponding to compounds of *Connarus regnellii* (Connaraceae) – Bark extract with GNPS [Colour figure can be viewed at wileyonlinelibrary.com]

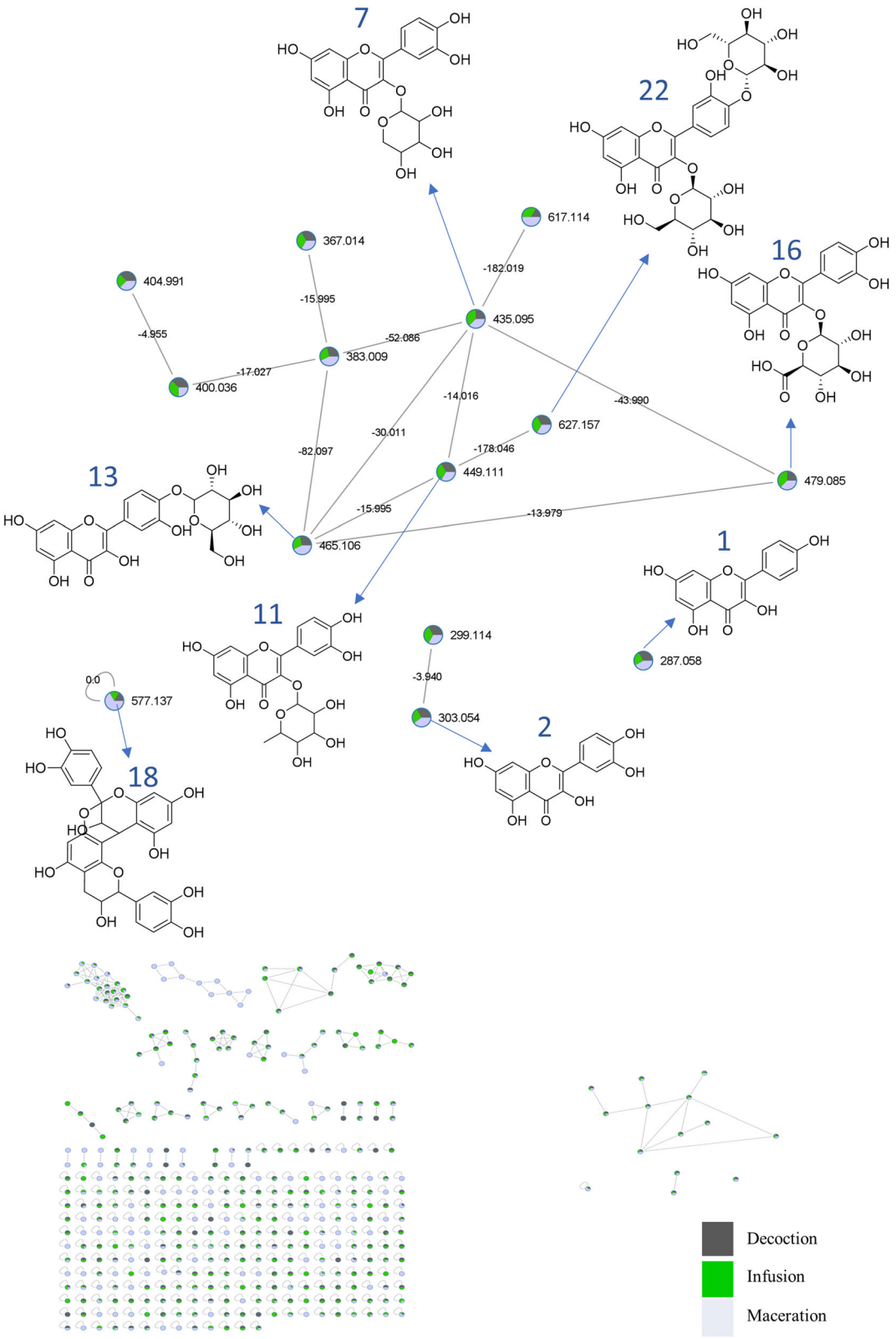

FIGURE 4 Clusters corresponding to compounds of *Connarus regnellii* (Connaraceae) – Leaf extract with GNPS [Colour figure can be viewed at wileyonlinelibrary.com]

pterocarpans, benzoquinones,[41] steroids, and terpenes.[40] In our recent review, we noted that this is one of the most important species of Connaraceae with high reported pharmacological potential.[14] In the barks of *C. suberosus* (CSB), the molecular network based on the similarity of MS/MS identified the presence of 243 precursor ions visualized as nodes in the molecular map (Figure 5), including 23 clusters

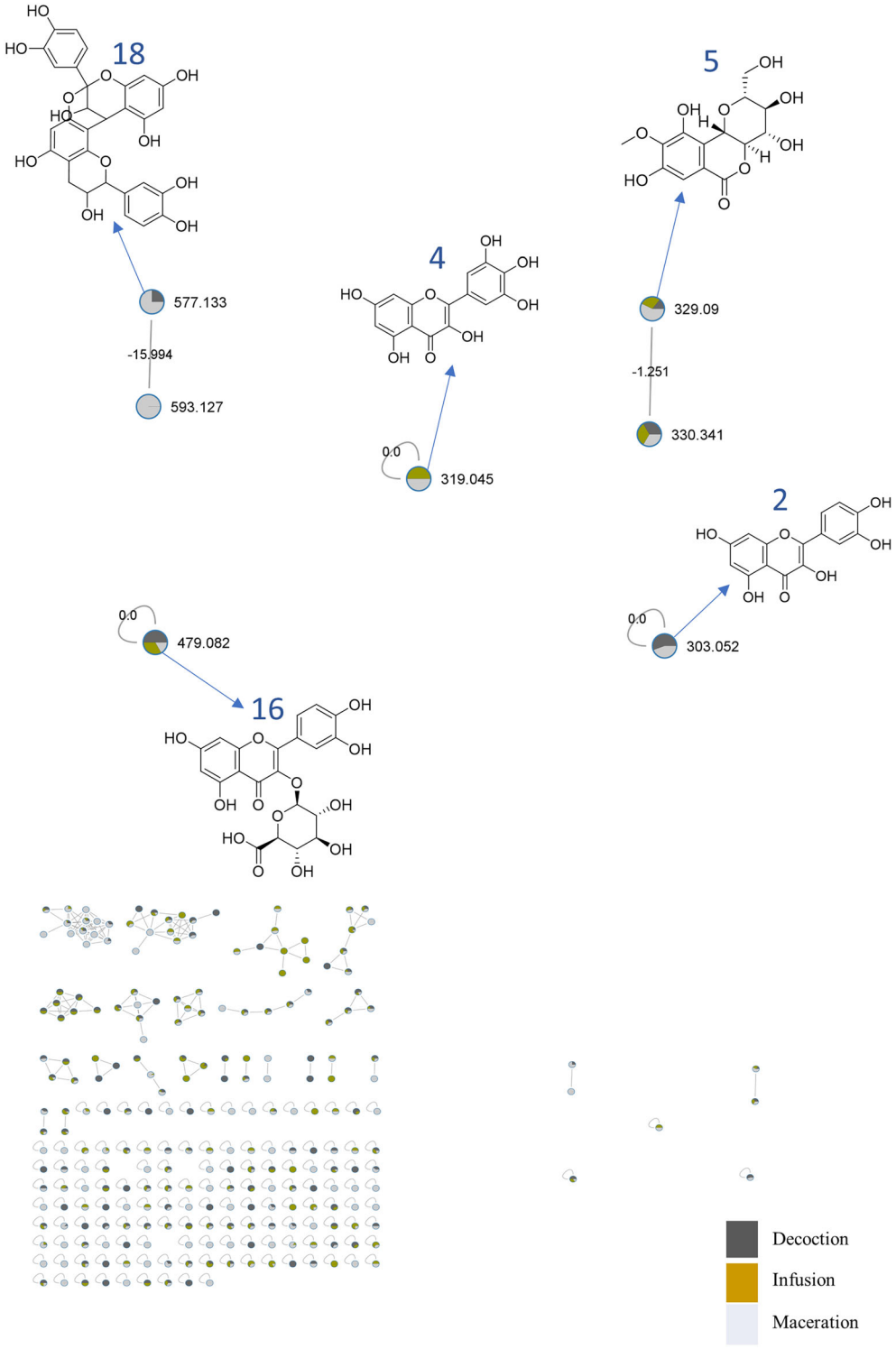

FIGURE 5 Clusters corresponding to compounds of *Connarus suberosus* (Connaraceae) – Bark extract with GNPS [Colour figure can be viewed at wileyonlinelibrary.com]

(node ≥ 2) and 220 unique nodes. The acid trihydroxybenzoic (bergenin), the aglycones quercetin and myricetin, and the heteroside quercetin-3-glucuronide, together with proanthocyanidin A2, were the compounds identified in CSB.

Quercetin-3-glucuronide, a derivative of quercetin, was the only heteroside identified in CSB. The metabolite bergenin was another compound seen in the bark of *C. suberosus*. The presence of bergenin has already been reported for other species of Connaraceae[42,43]; this compound is associated with antiallergic potential[44] and with beneficial effects on mitochondrial dysfunction.[45]

In *C. suberosus* leaves (CSL), GNPS analysis identified the presence of 480 precursor ions visualized as nodes in the molecular map (Figure 6), including 45 clusters (node ≥ 2) and 435 unique nodes. Among these nodes, 12 compounds could be identified; among them, kaempferol and quercetin were the aglycones identified. Additionally, eight heterosides were present, which included four compounds

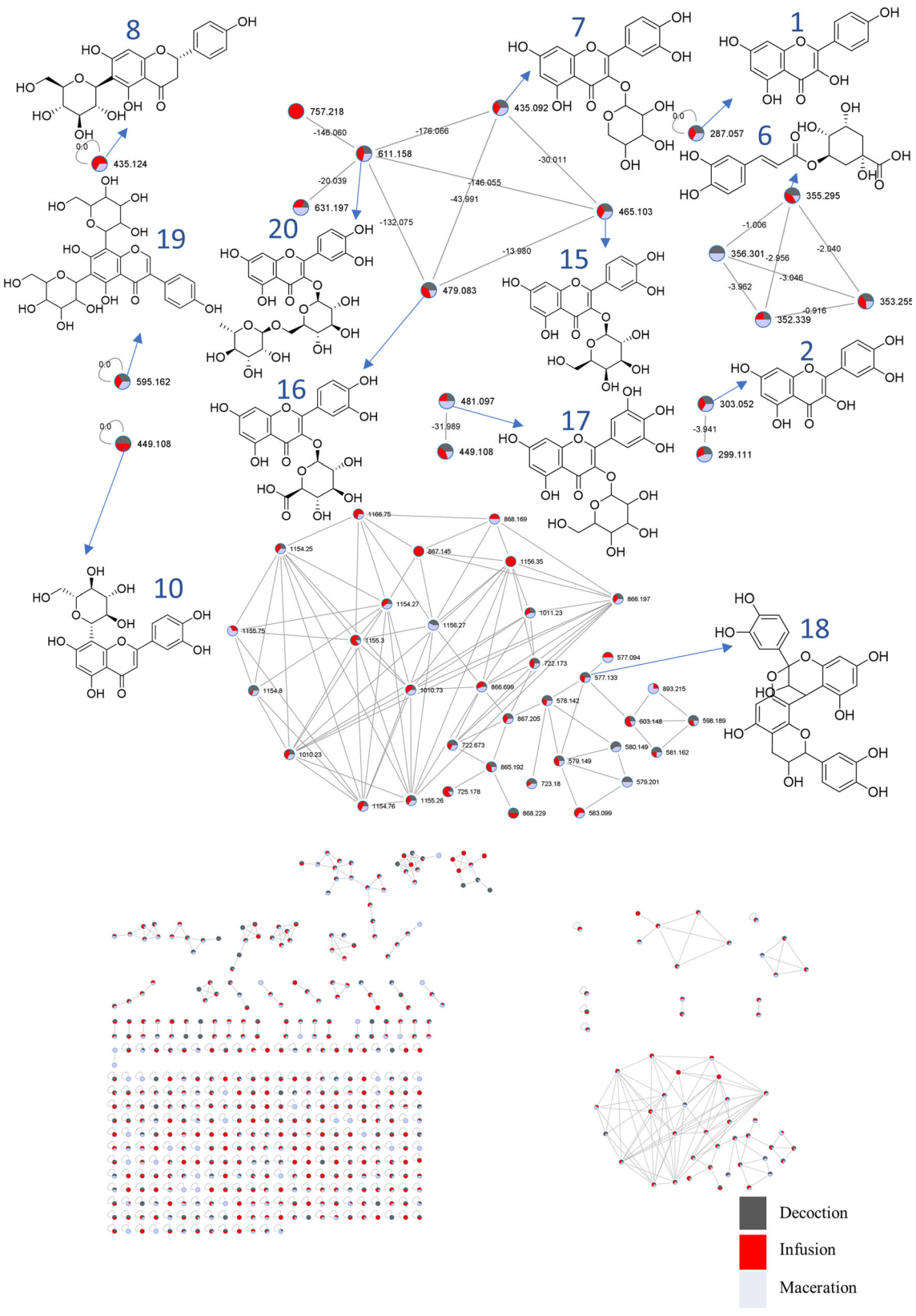

FIGURE 6 Clusters corresponding to compounds of *Connarus suberosus* (Connaraceae) – Leaf extract with GNPS [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 2 Chemical composition of four *Connarus* (Connaraceae) species through LC-ESI-MS/MS analysis in positive ion mode identified by the GNPS platform. Chemical compounds with corresponding retention times, molecular formulas, quasi-molecular ions, and key fragments are included

n°	Rt (min)	Molecular formula	$[M + H]^+$	MS fragments *m/z*	Compound	Species/ extractive methods*	References
1	67.0	$C_{15}H_{10}O_6$	287.0550	153 $[M - C_8H_5O_2]^+$, 213 $[M - C_2HO_3]^+$, 231 $[M - C_2O_2]^+$, 258 $[M - CO]^+$.	Kaempferol	CRL_{DIM} CSL_{DIM}	47,48,68
2	66.8	$C_{15}H_{10}O_7$	303.0499	153 $[M - C_8H_5O_3]^+$, 247 $[M - C_2O_2]^+$, 257 $[M - CHO_2]^+$, 285 $[M - OH]^+$.	Quercetin	CBL_{DIM} CNL_{DIM} CRB_{DIM} CRL_{DIM} CSB_{DM} CSL_{DIM}	48,49
3	64.9	$C_{15}H_{12}O_7$	305.0656	149 $[M - C_7H_7O_4]^+$, 153 $[M - C_8H_7O_3]^+$.	Dihydroquercetin	CNL_{IM}	69
4	65.0	$C_{15}H_{10}O_8$	319.0448	153 $[M - C_8H_5O_4]^+$, 165 $[M - C_7H_5O_4]^+$, 273 $[M - CHO_2]^+$, 301 $[M - OH]^+$.	Myricetin	CBL_{DIM} CNL_{DIM} CRB_{DIM} CSB_{IM}	48
5	14.8	$C_{14}H_{16}O_9$	329.0867	293 $[M - H_3O_2]^+$, 311 $[M - OH]^+$.	Bergenin	CSB_{DIM}	68
6	7.5	$C_{16}H_{18}O_9$	355.1024	163 $[M - C_{11}H_{11}O_3]^+$, 175 $[M - C_9H_9O_4]^+$.	Chlorogenic acid	CSL_{DIM}	70
7	65.8	$C_{20}H_{18}O_{11}$	435.0922	153 $[M - C_{13}H_{13}O_7]^+$, 247 $[M - C_{11}H_7O_3]^+$, 257 $[M - C_6H_9O_6]^+$, 285 $[M - C_5H_9O5]^+$, 303 $[M - C_5H_7O_4]^+$.	Quercetin-3-*O*-pentoside (Guaijaverin)	CNL_{DIM} CRL_{DIM} CSL_{DIM}	50
8	61.6	$C_{21}H_{22}O_{10}$	435.1286	285 $[M - C_9H_9O_3]^+$.	Naringenin-6-C-glucoside	CSL_{IM}	No reference
9	63.6	$C_{25}H_{31}N_3O_4$	438.2387	204 $[M - C_{14}H_{19}N_2O_2]^+$, 218 $[M - C_{13}H_{17}N_2O_2]^+$, 275 $[M - C_9H_8]^+$.	Dicoumaroyl spermidine	CNL_{DIM}	71
10	66.0	$C_{21}H_{20}O_{11}$	449.1078	153 $[M - C_{14}H_{15}O_7]^+$, 287 $[M - C_6H_9O_5]^+$.	Kaempferol 7-*O*-glucoside	CNL_{DIM} CSL_{DI}	72
11	66.1	$C_{21}H_{20}O_{11}$	449.1078	153 $[M - C_{14}H_{15}O_7]^+$, 257 $[M - C_7H_{11}O_6]^+$, 287 $[M - C_6H_9O_5]^+$, 303 $[M - C_6H_9O_4]^+$.	Quercetin-3-*O*-rhamnoside (quercitrin)	CNL_{DIM} CRL_{DIM}	73,74
12	65.6	$C_{20}H_{18}O_{12}$	451.0871	153 $[M - C_{13}H_{13}O_8]^+$, 263 $[M - C_4H_{11}O_8]^+$, 273 $[M - C_6H_9O_6]^+$, 319 $[M - C_5H_7O_4]^+$.	Myricetin-3-*O*-pentoside	CBL_{DM} CNL_{DIM}	No reference

(Continues)

TABLE 2 (Continued)

n°	Rt (min)	Molecular formula	$[M + H]^+$	MS fragments *m/z*	Compound	Species/ extractive methods*	References
13	65.7	$C_{21}H_{20}O_{12}$	465.1028	153 $[M - C_{14}H_{15}O_8]^+$, 247 $[M - C_{12}H_9O_4]^+$, 285 $[M - C_6H_{11}O_6]^+$, 303 $[M - C_6H_9O_5]^+$.	Quercetin-4′-*O*-glucoside	CRL_{DIM}	No reference
14	65.4	$C_{21}H_{20}O_{12}$	465.1028	153 $[M - C_{14}H_{15}O_8]^+$, 263 $[M - C_5H_{13}O_8]^+$, 273 $[M - C_7H_{11}O_6]^+$, 319 $[M - C_6H_9O_4]^+$.	Myricetin-3-*O*-rhamnoside (myricitrin)	CBL_{DIM} CNL_{DIM} CRB_{DIM}	[74]
15	65.7	$C_{21}H_{20}O_{12}$	465.1028	153 $[M - C_{14}H_{15}O_8]^+$, 285 $[M - C_6H_{11}O_6]^+$, 303 $[M - C_6H_9O_5]^+$.	Quercetin 3-*O*-galactoside (hyperin)	CSL_{DIM}	[11,73]
16	65.7	$C_{21}H_{18}O_{13}$	479.0820	153 $[M - C_{14}H_{13}O_9]^+$, 247 $[M - C_{12}H_7O_5]^+$, 257 $[M - C_7H_9O_8]^+$, 285 $[M - C_6H_9O_7]^+$, 303 $[M - C_6H_7O_6]^+$.	Quercetin-3-*O*-glucuronide (quercituron)	CBL_{DIM} CRL_{DIM} CSB_{DIM} CSL_{DIM}	[50,74,75]
17	64.1	$C_{21}H_{20}O_{13}$	481.0977	153 $[M - C_{14}H_{15}O_9]^+$, 273 $[M - C_7H_{11}O_7]^+$, 319 $[M - C_6H_9O_5]^+$.	Myricetin-3-*O*-hexoside	CBL_{DM} CNL_{DIM} CSL_{DIM}	[75]
18	43.6	$C_{30}H_{24}O_{12}$	577.1341	407 $[M - C_{12}H_9O]^+$, 425 $[M - C_{12}H_7]^+$, 451 $[M - C_7H_9O_2]^+$.	Proanthocyanidin A1 (procyanidin A1)	CBL_{DM} CRB_{DIM} CRL_{DIM} CSB_{DM} CSL_{DIM}	[11]
19	59.8	$C_{27}H_{30}O_{15}$	595.1657	559 $[M - H_3O_2]^+$.	5,7-Dihydroxy-3-(4-hydroxyphenyl)-6,8-bis[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one	CSL_{DIM}	No reference
20	65.9	$C_{27}H_{30}O_{16}$	611.1607	153 $[M - C_{20}H_{25}O_{12}]^+$, 273 $[M - C_{13}H_{21}O_{10}]^+$, 285 $[M - C_{12}H_{21}O_{10}]^+$, 303 $[M - C_{12}H_{19}O_9]^+$.	Quercetin 3-*O*-rutinoside (rutin)	CSL_{DIM}	[50,74]
21	65.9	$C_{28}H_{24}O_{16}$	617.1137	153 $[M - C_{20}H_{19}O_{12}]^+$, 319 $[M - C_{13}H_{13}O_8]^+$.	Gallomyricitrin	CBL_{DM}	No reference
22	62.5	$C_{27}H_{30}O_{17}$	627.1556	303 $[M - C_{12}H_{19}O_{10}]^+$, 465 $[M - C_6H_9O_5]^+$.	Quercetin 3,4′-diglucoside	CRL_{DIM}	No reference
23	60.7	$C_{28}H_{24}O_{17}$	633.1086	153 $[M - C_{21}H_{19}O_{13}]^+$, 171 $[M - C_{21}H_{17}O_{12}]^+$, 319 $[M - C_{13}H_{13}O_9]^+$.	Myricetin 3-*O*-beta-D-galactoside 6′′-*O*-gallate	CNL_{DIM}	No reference

The standards were used for confirmation of compounds 1, 2, 3, and 20 (Supporting Information, Figures S1, S2, S3s and S4).
All MS/MS fragments checked for the compounds were confirmed by CFM-ID analysis computed results (Supporting Information).
The acronyms referring to the species are followed by the letters D (decoction), I (infusion), and M (maceration) according to the occurrence of the compound in the extractive method.

derived from quercetin. Finally, chlorogenic acid, naringenin-6-C-glucoside, kaempferol 7-O-glucoside, proanthocyanidin A1, and the compound 5,7-dihydroxy-3-(4-hydroxyphenyl)-6,8-bis[3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]chromen-4-one can also be found in CSL.

For CSL, 10 out of 12 compounds are present in the three extractive methods (exceptions are kaempferol 7-O-glucoside compounds, detectable only in decoction and infusion, and naringenin-6-C-glucoside, which was present only in the infusion and maceration). In the cluster referring to the heterosides identified in CSL, the quercetin 3-galactoside has a 146 Da variation in relation to a rutin that has glucose and rhamnose sugar groups. Additionally, CSL also showed an extensive anthocyanidin cluster, among which the compound proanthocyanidin A1 can be identified.

In a recent review, it became clear that the number of chemical compounds known for the genus *Connarus* included a few dozen compounds reported for only five species[14]: *Connarus angustifolius*, *C. monocarpus* L., *C. paniculatus* Roxb., *C. semidecandrus* Jack, and *C. suberosus*. Here, we have identified the presence of at least 23 chemical compounds (Table 2), including flavonoids and their respective heterosides, most of which are unpublished for this plant genus, and increased the number of *Connarus* species whose chemical composition was studied from 5 to 8 species.

As seen in Table 2, most of the compounds identified for the species were identified between 60 and 66 minutes when methanol makes up 100% of the solvent, which indicates that these compounds have greater affinity for this solvent. In another study, the authors using the same mobile phase showed similar behavior in the analysis of compounds present in *Myrcia multiflora* (Lam.) DC leaves with the flavonoids guaijaverin (7), quercitrin (11), myricitrin (14), and hyperin (15).[46]

In this work, in addition to the annotation made from the GNPS platform and the confirmation of the identity of the metabolite conducted by spectral prediction (CFM-ID), the standards kaempferol, quercetin, dihydroquercetin, and rutin were used. For kaempferol, the fragments 153 [M – $C_8H_5O_2$]$^+$, 213 [M – C_2HO_3]$^+$, 231 [M – C_2O_2]$^+$, and 258 [M – CO]$^+$ could be verified with common ions between the primary standard and the samples, this being a fragmentation pattern similar to that observed in other works.[47,48] For quercetin the fragments 153 [M – $C_8H_5O_3$]$^+$, 247 [M – C_2O_2]$^+$, 257 [M – CHO_2]$^+$, and 285 [M – OH]$^+$ are verified, which are also among the most abundant in other studies.[48,49] Dihydroquecetin presented the fragments 149 [M – $C_7H_7O_4$]$^+$ and 153 [M – $C_{78}H_7O_3$]$^+$, in common between the standard and the samples, a fact that is probably related to the small concentration of this metabolite for *C. nodosus* species and, consequently, it presents low fragment ion intensity. For rutin, the fragment ions 153 [M – $C_{20}H_{25}O_{12}$]$^+$, 273[M – $C_{13}H_{21}O_{10}$]$^+$, 285 [M – $C_{12}H_{21}O_{10}$]$^+$, and 303 [M – $C_{12}H_{19}O_9$]$^+$ appeared among the most intense in common generated by the standard and the sample, which is also reported in the literature for this metabolite.[50]

In the following sections, we consider some of the identified compounds here presented, in order to discuss their pharmacological potential associated with these metabolites and the respective species.

3.1 | Quercetin

Quercetin (3,4,5,7-pentahydroxylflavone) (compound 2) is a flavonoid found in many species of plants[51] and was first reported for the genus *Connarus* in the form aglycone. In the GNPS analysis six fragment ions were considered for the annotation of all compounds in the molecular networks. Additionally, the identity confirmation of the compound quercetin can be verified through the prediction of the fragmentation spectrum in the CFM where three fragment ions (153, 257, and 285) were identified and a chemical structure was proposed (Supporting Information, Figure S6). Here, quercetin was found in all analyzed species and among the identified compounds it appeared as one of the main metabolites in CNL, CRB, CRL, and CSB. The presence of quercetin had already been identified in *Rourea*, namely in *R. coccinea*,[3,52] *R. induta*,[12,53] and *R. minor*.[54] Studies using a pharmacological approach of these Connaraceae species showed that quercetin is associated with antinociceptive activities,[12] antioxidant and hepatoprotective activities,[53] and an ameliorative effect.[3] In other plant families, the presence of quercetin has been associated with antioxidant and anti-inflammatory action, as reported by in vitro and in vivo studies,[55,56] and in vitro inhibition of α-glucosidase,[57] cardiac function benefits,[58] and antiviral activity.[59]

3.2 | Myricetin

Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone) (compound 4) also belongs to the flavone class. According to the annotation in the molecular network obtained from the GNPS, by the parameters already mentioned, myricetin had 5 fragment ions (153, 165, 273, 301, and 319) identified and proposed by CFM (Supporting Information, Figure S8). Myricetin was firstly identified among Connaraceae species and proved to be one of the main chemical constituents of CBL, CNL, and CRB. The pharmacological potential of myricetin was linked with inhibitory activity for α-glucosidase[57] and α-amylase enzymes.[60]

3.3 | Quercetin-3-*O*-pentoside

Quercetin-3-*O*-pentoside (guaijaverin) (compound 7) is a quercetin glycoside formed by the attachment of an arabinoside to the OH group at position 3 in ring C of the flavonoid structure.[59] In QTof-MS/MS analysis for the compound guaijaverin (Supporting Information, Figure S11), the most intense ion was 303.0469 [M + H]$^+$, which was compatible with the loss of the sugar moiety pentose (−132 Da). Through CFM, four fragment ions (247, 257, 285, and 303) were identified and proposed.

Guaijaverin was found in all extracts for CNL, CRL, and CSL. In Connaraceae, this component has already been described for *Rourea cuspidata*[11] and *R. induta*,[12] in which the compound was reported with antidiabetic effect in *R. cuspidata*[11] and an antinociceptive and hepatoprotective effect for the extracts of *R. induta*.[12,53] Antidiabetic

effects were demonstrated for guaijaverin from the extract of *Myrcia multiflora* in which aldose reductase and α-glucosidase inhibition were observed.[61] Anti-advanced glycation end-products and antioxidant activities corroborated the antidiabetic potential of this compound.[46]

3.4 | Myricetin-3-*O*- rhamnoside

Myricetin-3-*O*- rhamnoside (myricitrin) (compound 14) is a rhamnose glycoside derivative of myricetin and can be found in some plants.[62] Here, for the first time, we have demonstrated that this metabolite is one of the main constituents of CBL, CNL, and CRB. The QTof-MS/MS analysis of myricitrin (Supporting Information, Figure S18) produced the most intense ion at 319.0431 $[M + H]^+$, suggesting a neutral loss of the sugar moiety rhamnose (−146 Da). Additionally, in CFM four fragment ions (153, 263, 273, and 319) were identified and proposed. Using a pharmacological approach, myricitrin showed antifibrotic effects at the hepatic level, antioxidant and anti-inflammatory activities in in vivo models,[63] and in vitro aldose reductase and α-glucosidase inhibition.[61]

3.5 | Quercetin 3-*O*-galactoside

Quercetin 3-*O*-galactoside (hyperin) (compound 15) (Supporting Information, Figure S19) produced the most intense ion at 303.0468 $[M + H]^+$, suggesting a neutral loss of the sugar moiety hexose (−162 Da). In CFM confirmation three fragment ions (153, 285, and 303) were identified and proposed.

Hyperin was found in all CSL extracts. The presence of hyperin in Connaraceae has already been described for *Rourea cuspidata*,[11] *R. induta*,[12] and *R. minor*.[54] For the former and the latter, pharmacological studies using an in vivo model were able to demonstrate a hypoglycemic effect, which may be associated with the presence of this compound.[6,11] Hyperin was shown to be capable of inhibiting the formation of advanced glycation end-products, evidencing an adjuvant effect on the reversal of the pathogenesis of diabetic vascular complications.[64] Pharmacological approaches reported for this compound in Connaraceae include a hypoglycemic effect for the extract of *R. cuspidata*[11] and an antinociceptive and hepatoprotective effect for the extracts of *R. induta*.[12,53] In other plant families in which hyperin has also been reported as one of the chemical constituents, the inhibition of α-glucosidase, α-glucosidase,[57,60] and α-amylase has been reported.[60]

3.6 | Quercetin 3-*O*-glucunoride

Quercetin 3-*O*-glucunoride (compound 16) is a quercetin derivative consisting of a flavonoid attached to a beta-D-glucuronopyranosyl moiety at position 3 via a glycosidic linkage.[65] Quercetin 3-*O*-glucunoride was identified in CBL, CRL, CSB, and CSL. This finding is the first report of the presence of quercetin 3-*O*-glucunoride in Connaraceae. The QTof-MS/MS analysis of quercetin 3-*O*-glucunoride (Supporting Information, Figure S20) produced the most intense ion at 303.0499 $[M + H]^+$, suggesting a neutral loss of the moiety glucuronide (−176 Da). In CFM analysis, four fragment ions (247, 257, 285, and 303) were identified and proposed. The antioxidant and antidiabetic potential of quercetin 3-*O*-glucunoride isolated from *Euphorbia schimperi* C. Presl has been demonstrated in vitro.[66] Quercetin 3-*O*-glucunoride has been shown to inhibit angiotensin-converting enzyme (ACE)[59] and was able to promote proliferation and migration of murine neuronal cells in vitro.[67]

Here, we found that the main heterosides present in different species of *Connarus* are derived from quercetin and myricetin and are formed by glycosidic bonds between the aglycone nucleus and the sugar portion by the hydroxyl group of position 3 in ring C. Considering the list of identified metabolites, we found similar chemical profiles in our qualitative analysis. Among the heterosides here reported, the majority can be found with all three extraction methods tested. According to the literature, the hydrolysis of the sugar–aglycone bond can imply changes in the solubility of the compounds, their absorption, and consequently their biological effects.[51] However, it was not possible to identify significant changes in the chemical composition of the species using the methods tested in this work. Therefore, as the data suggest, the extractive methods endorsed by popular use have an adequate performance in the extraction of the main metabolites for these species.

For the first time, the present study reported the use of metabolomic analyses via spectral libraries of the GNPS platform in order to qualitatively identify the presence or absence of metabolites of pharmacological interest from *Connarus* subjected to different extractive processes. Among the four studied species, it was possible to identify the presence of 23 chemical compounds, most of which have associated pharmacological potential. Our results indicate that the flavonoids quercetin and myricetin and their heterosides are the main flavonoids identified in the species of *Connarus*. For the listed compounds, the main related activities reported in the literature include antioxidant and anti-inflammatory effects, as well as inhibitory effects on enzymes related to carbohydrate metabolism, which could be related to the potential application of these four species of Connaraceae. These findings provide support for future research in order to study the pharmacological activities of extracts obtained from these taxa, mainly with respect to possible applications as anti-inflammatory, antioxidant, and antiallergic agents and in diabetes control.

The use of the metabolomic tools of the GNPS platform allowed us to identify chemical compounds relatively quickly and efficiently from neglected plant species (*C. blanchetii*, *C. nodosus*, and *C. regnellii*), in addition to *C. suberosus*, whose limited number of compounds have already been described and whose pharmacological potential has been reported. The constructed molecular networks allowed to compare the qualitative profile of metabolites extracted in each of the three tested methods, in order to establish whether the extraction process is capable of promoting significant changes in the pattern of compounds obtained for each species.

We recommend that these species are investigated in depth in future pharmacological and toxicological essays. Finally, we conclude that *Connarus* species are potentially important as sources of bioactive molecules, calling the attention of the scientific community to carry out additional research in order to expand our knowledge of the pharmacological potential of this plant family, in accordance with the preservation and sustainable use of Brazilian biodiversity, while promoting the discovery of new therapeutic alternatives based on Brazilian flora.

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DECLARATION OF COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this article.

AUTHOR CONTRIBUTIONS

Luis Fernando N. A. Paim and Paulo R. dos Santos conducted the chemical analyses and wrote the manuscript. Cássio A. P. Toledo and Joicelene R. L. da Paz collected and identified the plants and assisted in the modification and adaptation of the text. Luana Minello assisted in the modification and adaptation of the text. Sidnei Moura, Mirian Salvador, and Vinicius C. Souza made the final revision of the manuscript. All authors approved the final submitted version of the manuscript.

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SUPPORTING INFORMATION

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Capítulo 3

Chemical Composition, Antioxidant and Anti-AGEs Activities in four species of the Connaraceae Family

Chemical Composition, Antioxidant and Anti-AGEs Activities in four species of the Connaraceae Family

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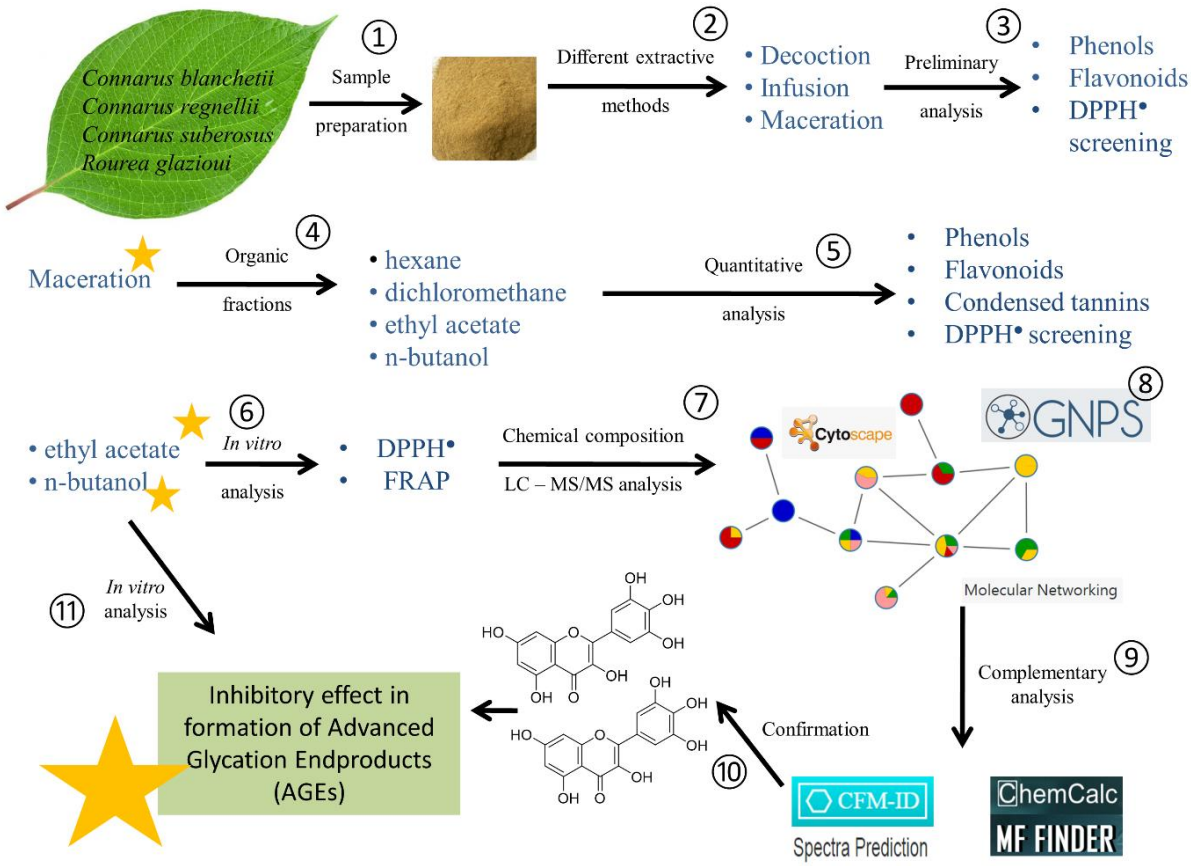

ABSTRACT

In order to discover new perspectives in the treatment of diabetes complications we propose to study the potential of four species of the Connaraceae family. C*onnarus blanchetii*, *C. regnellii*, *C. suberosus* and *Rourea glazioui* were submitted to different extractive methods and sequentially detailed in a preliminary phytochemical investigation. Then, after selecting the maceration method, organic fractions were obtained with the quantitative determination of metabolites where the ethyl acetate and n-butanol fractions presented the best results and in which the antioxidant potential was determined. The anti-AGEs activity was evaluated for the extracts with the best combination of the previous results. Finally, the chemical composition was determined through molecular networks and other bioinformatics tools where 29 specialized metabolites, mostly derivatives of quercetin and myricetin, could be identified.

Keywords: Connaraceae, Extractive methods, LC-MS/MS, Antioxidant, Advanced Glycation End Products

Members of Connaraceae family are mainly distributed in tropical areas, comprising 12 genera and about 200 plant species [1], 39 of which with associated pharmacological potential [2]. The use of Connaraceae plants as source in traditional medicine encompasses a wide range of applications, including the treatment of *Diabetes Mellitus* (DM), reported to some species of the genera *Cnestis*, *Connarus* and *Rourea* [3–6]. Studies reporting antidiabetic activities comprise preparations obtained from *Cnestis ferruginea* DC. [3], *Rourea coccinea* (Schumach. & Thonn.) Benth. [4,7]., *Rourea cuspidata* Benth. ex. Baker [6] and *Rourea minor* (Gaertn.) Alston [5,8]. In Connaraceae the hypoglycemic activity was measured in rats whose diabetes was induced by streptozotocin or alloxan, with the main objective being to evaluate the possible reduction in glycemia promoted by the different extracts [3,4,6]. Reviewing the potential of the chemical composition of the Connaraceae species we found that several flavonoids show glycation inhibitory and antioxidant potential [2,9]. Thus, we believe that the beneficial effects of Connaraceae metabolites in controlling DM may not only be associated with the hypoglycemic effect, extending to inhibition of protein glycation and reduction of oxidative stress.

The DM is a chronic disorder caused by elevated blood glucose that affects the metabolism of carbohydrates, lipids and proteins [10]. The hyperglycemia is the factor that triggers long-term complications, causing oxidative damage followed by imbalance between the production of reactive oxygen species (ROS) or the antioxidant defense mechanisms [11–13]. Evidence suggests that diabetic patients are more vulnerable to oxidative stress because they have a higher production of ROS than patients who do not have the disease [13]. In diabetes, mitochondrial processes in oxidative phosphorylation represent the main source of free radicals contributing to non-enzymatic glycation of proteins, glucose oxidation, increased lipid peroxidation, damage to enzymes and increased insulin resistance [11]. Evidence suggests that even patients treated with oral hypoglycemiants are susceptible to oxidative stress since these drugs cannot reverse all changes caused by hyperglycemia [14]. Clinical studies showed that

antioxidant treatments with vitamins C and E and α-lipoic acid provided positive results in preventing complications of diabetes [12]. Among the many pathophysiological changes resulting from DM, the accelerated generation of advanced glycation end products (AGEs) associated with chronic hyperglycemia leads to cell and tissue damage observed in the course of DM [15,16]. AGEs are a heterogeneous group of products that are irreversibly formed through non-enzymatic glycation and oxidation of proteins, nucleic acids and lipids which can promote cell death and contribute to the development of diabetic complications [17] including nephropathy, neuropathy and retinopathy [16].

In this line, researchers have shown phenolics compounds, manly flavonoids are active against the inhibition of AGE formation [13,16,18]. The last molecules are widely reported as metabolites in Connaraceae [6,19–21]. In this context, considering the pharmacological potential of this family of plants, this work aims to analyze the chemical composition and the antioxidant and anti-AGEs potential of four species: *Connarus blanchetii* Planch.; *Connarus regnellii* G. Schellenb.; *Connarus suberosus* Planch and *Rourea glazioui* G. Schellenb. In the first step, all species were subjected to ethanolic maceration followed by extraction by different solvents where the metabolites were quantified. Sequentially, from the richest fractions, the antioxidant and anti-AGEs activities were evaluated and the chemical compounds analyzed by LC-MS/MS followed by identification via Global Natural Product Social (GNPS) and complemented by other bioinformatics platforms.

RESULTS AND DISCUSSION

Different extractive methods and preliminary analysis

The use of medicinal plants has increased with the dissemination of ethnopharmacological knowledge, the addition of related scientific information, as well as related to the cultural issue that leads to greater use in specific regions of the planet [22–24]. In this sense, these have usually been used in the form of infusions, decoction and macerated,

among other [25] It is no different for species of the Connaraceae family which has been used as: decoction [26–28], infusion [29] and maceration [30].

Thus, in order to identify the most effective process for the production of phenolic compounds as a first step we have determined the total polyphenol and flavonoid content from preparations carried-out by decoction, infusion and maceration. Additionally, a (2,2-diphenyl-1-picrylhydrazyl radical) $DPPH^{\bullet}$ scavenging activity screening was conducted. The Figure 1 a-c report the results.

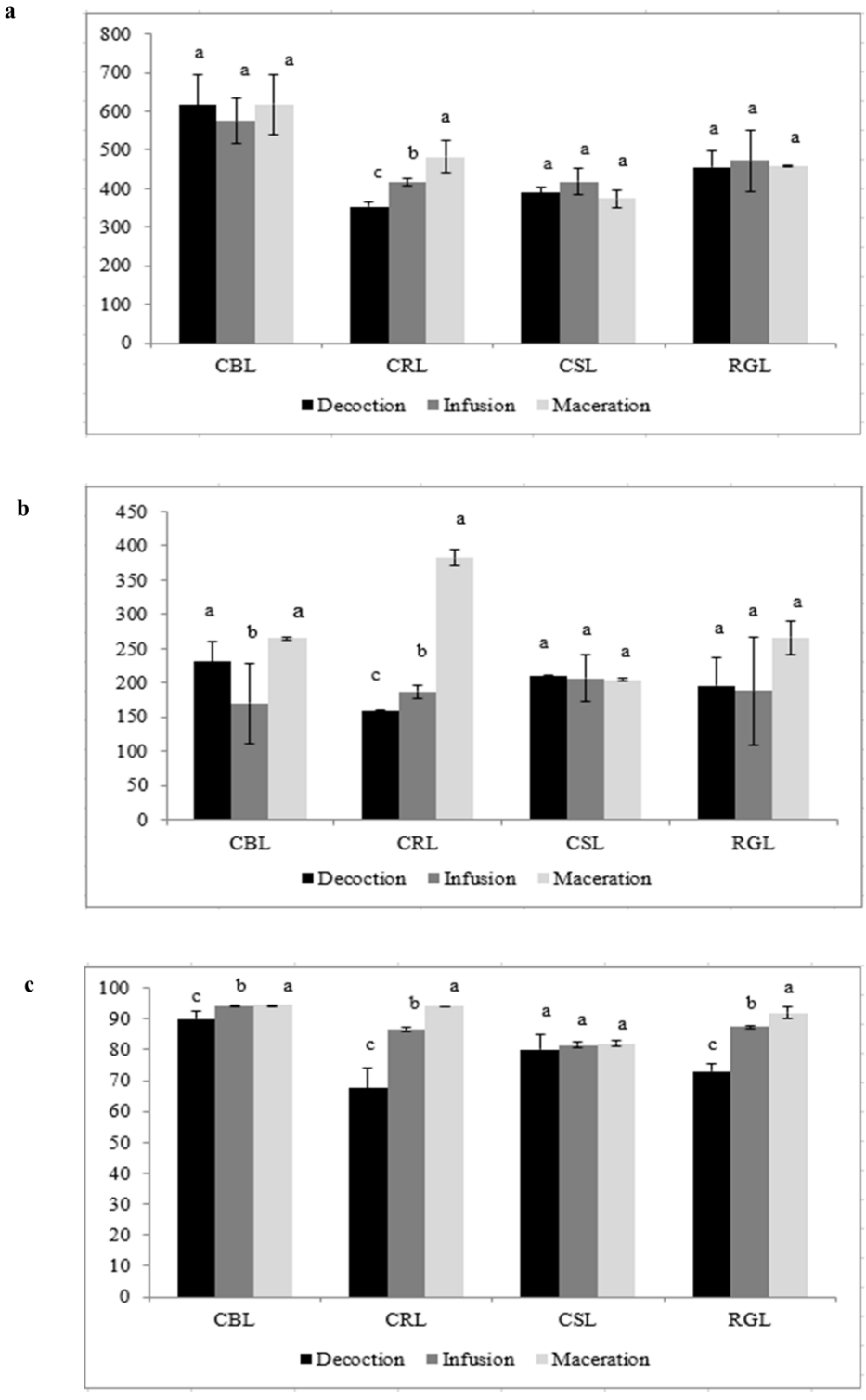

Figure 1: Analysis with crude extracts: a) total phenols (µg gallic acid. Eq./mg dry extract); b) flavonoids (µg quercetin. Eq/mg dry extract) and c) screening SC% with DPPH$^{\bullet}$40 µg/mL and extract 125 µg/mL Where: CBL = *C. blanchetii* leaves; CRL = *C. regnellii* – leaves; CSL = *C. suberosus* – leaves and RGL = *R. glazioui*. The means followed by various letter were significantly different at $p \leq 0.05$ according the Kruskal-Wallis test.

The results the analysis of total polyphenol contents, Figure 1a, show that for CRL, maceration had a better quantitative profile, while for the other species the method did not produce significant changes. For the total flavonoids Figure 1b in CBL and CRL, maceration was the method with the best performance. In the DPPH• screening, Figure 1c, for CBL, CRL and RGL, maceration presented the highest percentage of radical scavenging. In Connaraceae in our previous article we reported some changes in the qualitative profile of compounds for four species of the genus *Connarus* [9] however from the quantitative point view, as far as we know, no working reporting comparation between methods of extraction have been conducted. In other genera and plant species differences in the quantitative profile of metabolites are reported [25,31]. For *Dicksonia sellowiana* Hook., polyphenols, flavonoids, protoanthocyanins extracted by decoction, infusion and maceration show different quantitative profiles which are associated with changes in the antioxidant potential measured by DPPH• [25]. In *Syzygium cumini* (L.) Skeels different extractive processes produce changes in the quantitative profile of total polyphenols and activity against DPPH• [31]. Different authors have reported that yields phenolics depend on different factors including the type of solvent used, the plant matrix and the duration of the extractive process employed [25,32,33]. However, in accordance with the literature, we believe that the proper selection of the extraction method is essential for the advancement of research processes as well as for the improvement of the biological effect associated with different plant species [34]. In this way, from these results, mainly based on DPPH• screening, we have selected maceration as an extractive method to continue the experiments.

Organic fractions and new quantitative analysis

In sequence we propose the polyphenols content evaluation from the selected method with different organic solvents Figure 2. In this stage the extract carried-out from maceration was sequentially fractioned using hexane, dichloromethane, ethyl acetate and *n*-butanol. In addition to quantitative analysis a new screening of antioxidant activity with DPPH$^{\bullet}$ was performed.

For the fractionated macerate, the results of the quantification tests of total polyphenols, Figure 2a, and condensed tannins show that the more polar fractions of the solvents (ethyl acetate and n-butanol) have shown the highest amount of these two classes of phenolic compounds. In the analysis of the scavenging % (SC%) whit DPPH$^{\bullet}$ Figure 2d the elimination percentage was equally higher for the ethyl acetate and n-butanol fractions. In the quantification of total flavonoids Figure 2b there is a better performance for the ethyl acetate fraction for CBL, CRL and CSL species and for the dichloromethane fraction in RGL. The behavior regarding the content of phenolic acids, flavonoids and tannins associated with sequential fractionation with organic solvents for most plant species has been shown as follows ethyl acetate ≥ n-butanol > dichloromethane/chloroform > hexane [35–38] this behavior being compatible with that observed for the Connaraceae species studied. Thus, combining the results obtained for total polyphenols, condensed tannins and the percentage of scanning with DPPH$^{\bullet}$ observed for different fractions, we conducted new experiments to establish a profile regarding the antioxidant potential of the ethyl acetate and n-butanol fractions.

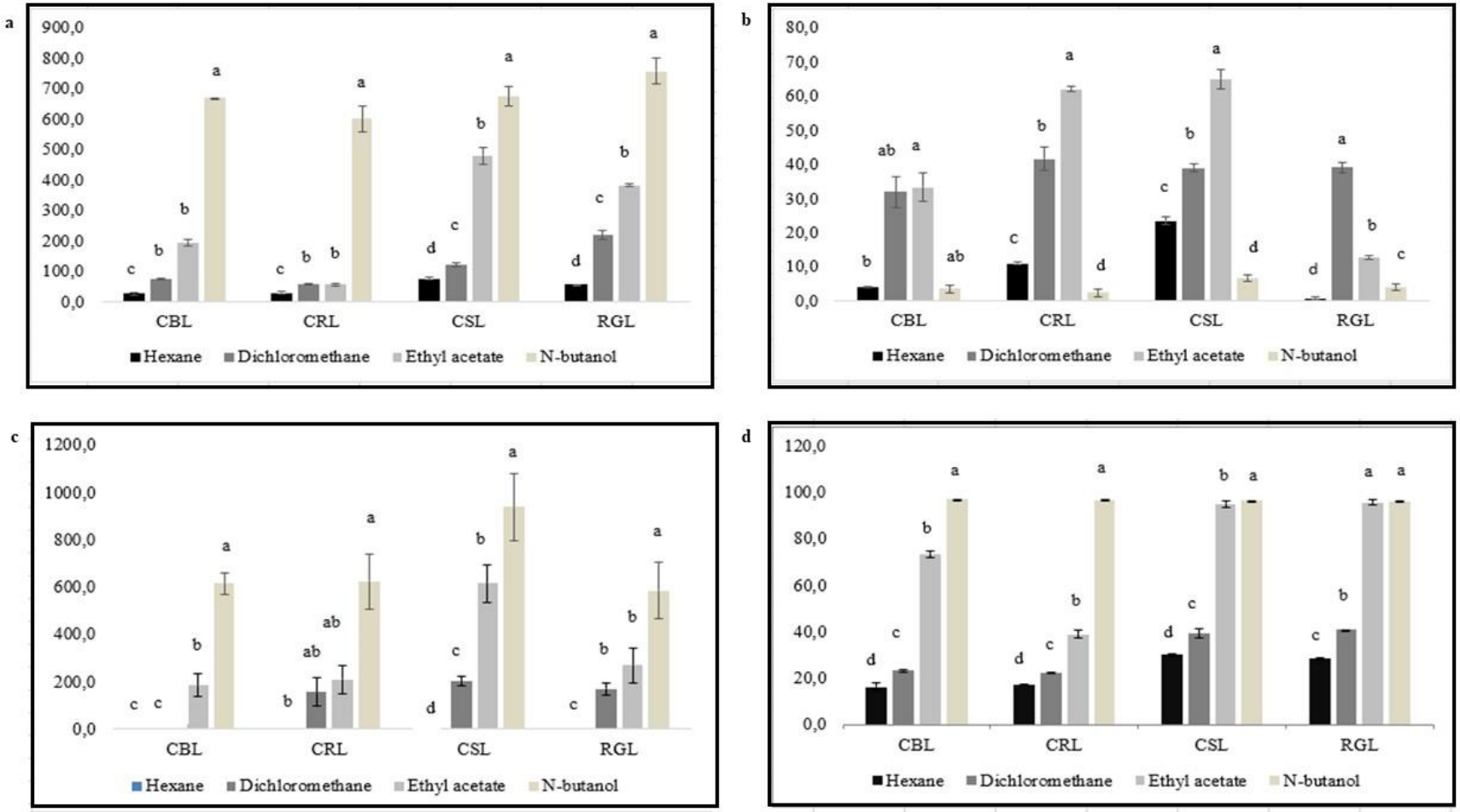

Figure 2: Analysis with fractional extracts: a) total phenols (µg gallic acid. Eq./mg dry extract); b) flavonoids (µg quercetin. Eq./mg dry extract), c) condensed tannins (µg catequin Eq./mg dry extract) and d) screening (SC%) with DPPH•40 µg/mL with dry extract 125 µg /mL. Where: CBL = *C. blanchetii* leaves; CRL = *C. regnellii* – leaves; CSL = *C. suberosus* – leaves and RGL = *R. glazioui*. The means followed by various letter were significantly different at $p \leq 0.05$ according the Kruskal-Wallis test.

In vitro analysis antioxidant potential

The evaluation of the antioxidant potential table 1 of the ethyl acetate and n-butanol fractions was studied using the DPPH• experiments where the respective SC_{50} measured in µg/mL of dry extract (µg.ext.dry/mL) were determined. In addition, Ferric Reducing Antioxidant Power (FRAP) assay whose results were expressed in µg/mL of dry extract equivalent to 1000 mM of ferrous sulphate ($FeSO_4 .7H_2O$) was conducted.

Table 1: *In vitro* antioxidant activity via DPPH• and FRAP assays for the ethyl acetate and n-butanol fractions.

	(DPPH•) radical scavenging		(FRAP) ferric reducing antioxidant power	
	Ethyl acetate	n-Butanol	Ethyl acetate	n-Butanol
Entry	SC_{50} µg.ext.dry/mL		µg.ext.dry /mL eq. 1.000 mM $FeSO_4$•$7H_2O$	
CBL	146.6	38.51	277.4	75.6
CRL	601.8	40.18	1477.0	81.3
CSL	80.7	31.09	183.7	72.5
RGL	92.4	39.35	78.9	143.2

CBL = *C. blanchetii* leaves; CRL = *C. regnellii* – leaves; CSL = *C. suberosus* – leaves and RGL = *R. glazioui* – leaves.

The evaluation of DPPH• assay showed that for all species the *n*-butanol fraction showed a better performance in comparison with the ethyl acetate fraction. In the FRAP assay, CBL, CRL and CSL, showed the best iron-reducing power, while in RGL, ethyl acetate fraction had the best result. Antioxidant assays show different reaction behaviors against the chemical nature and solubility of molecules involved in the reaction [39] all this methods have some limitations and show different tendencies to react against different classes of phenolic compounds [40]. Despite that the correlation between the high levels in the total phenols and flavonoids with a better performance of antioxidant activity is reported for various species including *Lagenaria siceraria* (Molina) Standl. [35] *Anthemis praecox* Link [36], *Ononis mitissima* L., [37] and *A. edulis* and *C. vernalis* where the ethyl acetate and n-butanol fractions showed to be the most promising [41]. Therefore, considering the results obtained in the metabolite quantification assays with the antioxidant activity performance for these Connaraceae taxa, we

chose to analyze the antiglycation potential of the n-butanol fraction of all species in addition to the ethyl acetate fraction of *R. glazioui*.

Inhibitory effect in Advanced Glycation End products (AGEs)

The evaluation of the inhibitory activity of protein glycation (IAPG) was evaluated for the oxidative and non-oxidative pathways Table 2. For IAPG were tested the extracts CBLnb, CSLnb and RGLea at a 100 µg/mL, which showed values greater than 50% inhibition and in these the IC_{50} were determined. In this line, the best result was for RGLea with IC_{50} of 36.5 µg/mL, which is better than observed for the standard quercetin.

In the assessment of the inhibitory activity for the non-oxidative pathway, all extracts showed an inhibition percentage greater than 50% when tested at 100 µg/mL. In this case, all IC_{50} were determined and RGLea had the most potent effect 4.5 µg/mL, this result being lower than that observed for the standards quercetin 21.2 µg/mL and aminoguanidine 36.3 µg/mL. For CBL, CRL, CSL and RGL the IC_{50} were 8.4, 9.4, 22.1 and 13.7 µg/mL respectively.

Table 2: Anti-AGE activities of the dry fractional extracts of the Connaraceae leaves.

Samples	Oxidative glycation inhibition		Non-oxidative glycation inhibition	
	Inhibitory effect (%) at 100 µg/mL	IC_{50} µg/mL	Inhibitory effect (%) at 100 µg/mL	IC_{50} µg/mL
CBLnb	54.0 ±1.2	78.0 (71.6 to 84.9)	96.5±1.5	8.4 (5.5 to 12.7)
CRLnb	47.3±1.0	NT	95.5±0.5	9.4 (6.2 to 14.2)
CSLnb	58.5±0.7	54.9 (50.2 to 60.0)	96.7±0.9	22.1 (13.4 to 36.3)
RGLea	77.6±1.2	36.5 (34.0 to 39.3)	93.7±1.2	4.5 (3.0 to 6.8)
RGLnb	49.1±2.2	NT	98.5±0.7	13.7 (7.6 to 24.8)
Naringenin	33.3±5.0	NT	4.6±0.6	NT
Quercetin	58.4±1.0	46.3 (37.4 to 57.3)	96.0±0.1	21.2 (12.8 to 35.2)
Acarbose	NT	NT		NT
Aminoguanidine	NT	NT	77.4±2.2	36.3 (29.8 to 44.3)

CBL = *C. blanchetii* leaves; CRL = *C. regnellii* – leaves; CSL = *C. suberosus* – leaves and RGL = *R. glazioui* – leaves, nb = n-butanol and ea = ethyl acetate. NT- Not tested

The search for chemical compounds that can inhibit the protein glycation process can bring great benefits to the health of diabetic patients. Some plant species, from other botanical families, have already had their anti-AGEs effects demonstrated, such as *Ilex paraguariensis* A.St.-Hil. [18], *Eugenia punicifolia* (Kunth) DC [42] and *Myrcia multiflora* (Lam.) DC [43]. In Connaraceae for the species *C. ferruginea*, *in vitro* model, it was established that the methanol extract of the leaves at a concentration of 30 µg/mL was able to reduce by 80% the glycation of human red blood cells, a result similar to the effect shown by the flavonoid quercetin tested at the same concentration [44].

Chemical composition

In order to try to establish the profile of chemical components which are involved with the biological effects, we study the n-butanol fraction of all species in addition to the ethyl acetate fraction of RGL. The data obtained by LC-MS/MS analysis were analyzed in the GNPS platform, using the molecular networking tool. Sequentially the identification tentative was complemented by competitive fragmentation analysis where the fragment ions compatible with metabolites were proposed via spectral prediction (SP) in Competitive Fragmentation Modeling for Metabolite Identification CFM-ID [45,46]. In last step the search of the respective molecular formulas of the fragmentation ions compatible between the fragmentation produced in the MS/MS analysis with the SP were obtained in ChemCalc [47]. The molecular network with all compounds identified is presented in Figure 3.

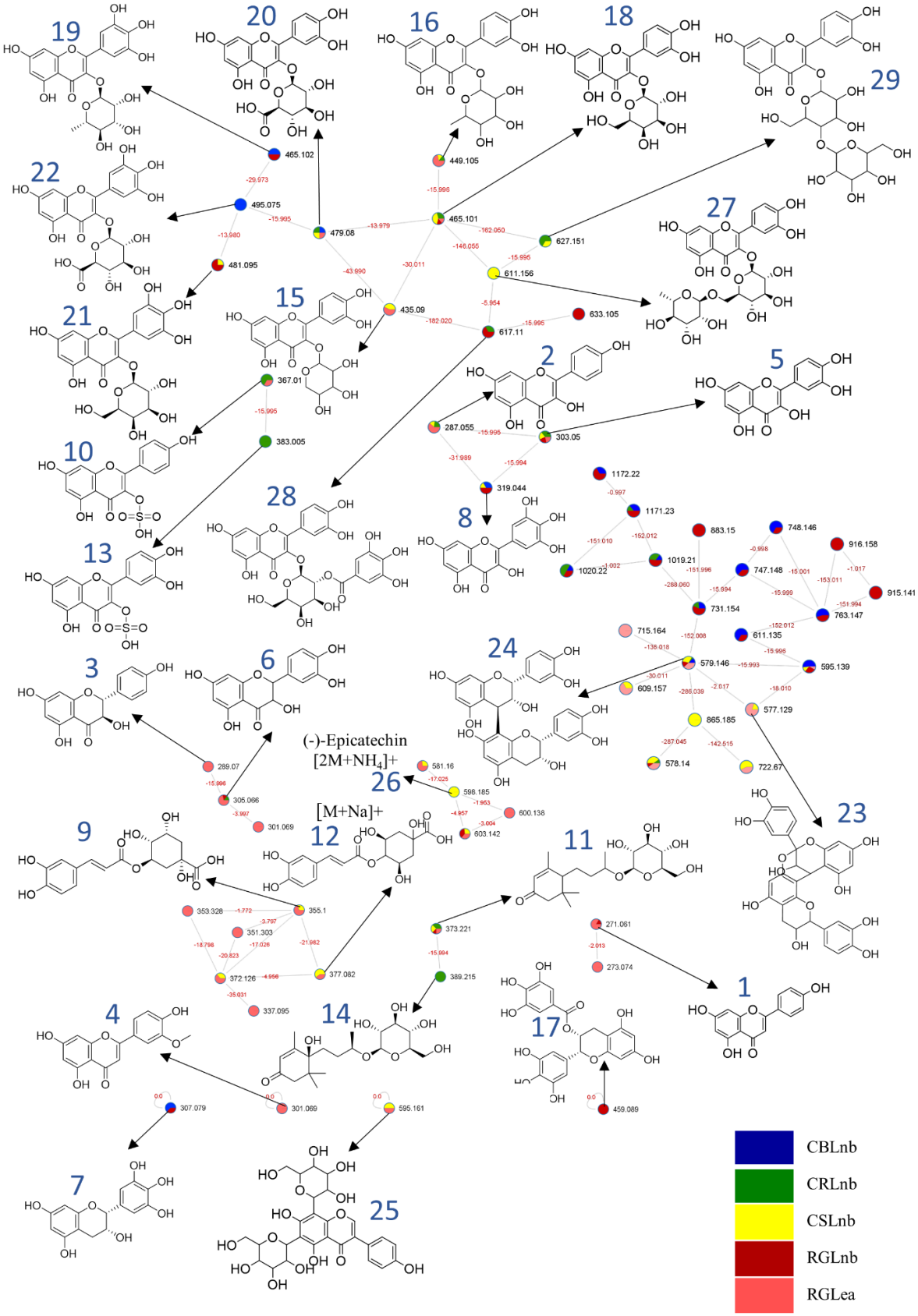

Figure 3: Comparative analysis of n-butanol (CBL, CRL, CSL and RGL) and ethyl acetate (RGL) fractions of the maceration in Connaraceae species using LC-MS/MS-based molecular networking. CBL = *C. blanchetii* leaves; CRL = *C. regnellii* – leaves; CSL = *C. suberosus* – leaves and RGL = *R. glazioui.*

Combining the tools, it was possible to propose in the molecular network the identification of 29 compounds. Among them the flavanols kaempferol $[M + H]^+$ *m/z* 287.0550 (compound 2), quercetin $[M + H]^+$ *m/z* 303.0500 (compound 5) and myricetin $[M + H]^+$ *m/z* 319.0440 (compound 8) form a single cluster with three nodes which are separated by a mass variation of 16 Daltons (Da), this being compatible with the mass referring to an oxygen atom among the chemical formulas of these metabolites as search made by MF finder from ChemCalc. Kaempferol and quercetin are present in *C. regnellii*, *C. suberosus* and *R. glazioui*, whereas myricetin has been identified in C. *blanchetii*, *C. suberosus* and *R. glazioui*. In our previous article, we had already described kaempferol, quercetin and myricetin in crude extracts obtained from four taxa of the genus *Connarus* [2]. The fragmentation obtained for kaempferol, quercetin and myricetin can be visualized in the figure S2, S5 and S8 (Supporting information) where the measured and predicted fragments are identified together with the chemical formulas to which they refer.

In the cluster referring to glycosylated flavonoids eleven nodes represent heterosides for these species where ten had their proposed identities. Quercetin-3-*O*-pentoside (compound 15), ion $[M + H]^+$ *m/z* 435.0900 is one of the metabolites associated with CSLnb and RGLea, being a glycosylated flavonoid derived from quercetin, genin fragment ion *m/z* 303.0473 figure SX (Supporting information), whose presence in Connaraceae has already been identified in *Rourea cuspidata* Benth. ex Baker [6] and in crude extracts in *Connarus nodosus* Baker, *C. regnellii* and *C. suberosus* [9]. The molecular ion $[M + H]^+$ *m/z* 449.1050 is compatible with quercetin-3-*O*-rhamnoside (compound 16), checked in CRLnb, CSLnb and RGLea, which was identified by the genin fragment ion *m/z* 303.0481 figure S15 (Supporting information). In quercetin-3-*O*-rhamnoside two others fragment ions *m/z* 153 and 287 measured in the mass spectrum are compatible with the predicted fragmentation of this metabolite. Through the molecular network it is possible to verify that quercetin-3-*O*-rhamnoside is distanced from

quercetin-3-*O*-galactoside (compound 18), other quercetin derivative, by 16 Da, which is compatible with an oxygen atom. Quercetin-3-*O*-galactoside is present in CRLnb, CSLnb and RGLea/nb this compound was described Connaraceae for *Rourea induta* Planch [48], *R. cuspidata* [6] and *C. suberosus* [9]. Myricetin-3-*O*-rhamnoside (compound 19), molecular ion $[M + H]^+$ *m/z* 465.1020 is one of the metabolites associated with CBLnb and RGLnb, being a glycosylated flavonoid derived from myricetin, genin fragment ion *m/z* 319.0438 figure S19 (Supporting information). In our previous work we described myricetin-3-*O*-rhamnoside in crude extracts for C. *blanchetii* and *C. nodosus* [9]. The other metabolites in this cluster were identified as quercetin-3-*O*-glucuronide molecular ion $[M + H]^+$ *m/z* 479.0800 (compound 20) for CBLnb, CRLnb, CSLnb and RGLea, myricetin-3-*O*-galactoside molecular ion $[M + H]^+$ *m/z* 481.0950 (compound 21) for CSLnb and RGLnb, myricetin-3-*O*-glucuronide molecular ion $[M + H]^+$ *m/z* 495.0750 (compound 22) for CBLnb, quercetin-3-*O*-rutinoside molecular ion $[M + H]^+$ *m/z* 611.1560 (compound 27) for CSLnb, quercetin 3-(2-galloylglucoside) molecular ion $[M + H]^+$ *m/z* 617.1100 (compound 28) for CRLnb and RGLnb and quercetin 3,4'-diglucoside molecular ion $[M + H]^+$ *m/z* 627.1510 (compound 29) for CRLnb and CSLnb.

Two other metabolites whose identity can be proposed were kaempferol-3-*O*-sulfate $[M + H]^+$ *m/z* 367.0100 (compound 10) and quercetin-3-*O*-sulfate $[M + H]^+$ *m/z* 383.0050 (compound 13) which form a cluster of two nodes whose mass difference is 16 Da. For these metabolites, the respective mass spectra produced the fragment ions *m/z* 287 figure S10 (Supporting information) and *m/z* 303 figure S13 (Supporting information) and as the most intense, respectively.

In addition to the compounds already mentioned several others could be identified among the species among the main: apigenin $[M + H]^+$ *m/z* 271.0610 (compound 1) for RGLea/nb, dihydroquercetin $[M + H]^+$ *m/z* 305.0660 (compound 6) for CRLnb and RGLea/nb, epigallocatechin $[M + H]^+$ *m/z* 307.0790 (compound 7) for CBLnb and RGLnb , chlorogenic

acid $[M + H]^+$ *m/z* 355.100 (compound 9) for CSLnb and RGLea, protoanthocyanidin A1 $[M + H]^+$ *m/z* 577.1290 (compound 23) for CSLnb and RGLea and $[M + H]^+$ *m/z* 579.1460 procyanidin B2 (compound 24) for CBLnb, CSLnb and RGLea/nb In summary this work the identity of 29 compounds among the different species of Connaraceae can be proposed more information can be accessed in the table 3 and in the support information file.

Table 3: Chemical composition of four Connaraceae species through LC-ESI-MS/MS in positive ion mode analysis identified by GNPS platform.

Entry	Rt (min.)	Molecular formula	*m/z* exact	Adduct	Fragmentation pathway	Compound	Species fractions	References
1	67.2	$C_{15}H_{10}O_5$	271.0601	$[M + H]^+$	119 $[M – C_{11}H_3O]^+$, 145 $[M – C_5H_5]^+$, 153 $[M – C_8H_5O]^+$.	Apigenin	RGLnb, RGLea.	49,50
2	66.8	$C_{15}H_{10}O_6$	287.0550	$[M + H]^+$	153 $[M – C_8H_5O_2]^+$, 269 $[M – OH]^+$.	Kaempferol	CRLnb, CSLnb, RGLea.	8,51,52
3	65.6	$C_{15}H_{12}O_6$	289.0707	$[M + H]^+$	121 $[M – C_{12}H_7O]^+$, 153 $[M – C_8H_7O_2]^+$.	Dihydrokaempferol	RGLea.	50
4	67.1	$C_{16}H_{12}O_6$	301.0707	$[M + H]^+$	153 $[M – C_9H_7O_2]^+$.	Chrysoeriol	RGLea.	
5	65.0	$C_{15}H_{10}O_7$	303.0499	$[M + H]^+$	137 $[M – C_8H_5O_4]^+$, 153 $[M – C_8H_5O_3]^+$, 257 $[M – CHO_2]^+$.	Quercetin	CRLnb, CSLnb, RGLnb, RGLea.	52,53
6	64.7	$C_{15}H_{12}O_7$	305.0656	$[M + H]^+$	123 $[M – C_8H_5O_5]^+$, 153 $[M – C_8H_7O_3]^+$.	Dihydroquercetin	CRLnb, RGLnb, RGLea.	50,54
7	25.2	$C_{15}H_{14}O_7$	307.0812	$[M + H]^+$	139 $[M – C_{12}H_7O]^+$, 151 $[M – C_9H_7O]^+$, 163 $[M – C_6H_7O_4]^+$.	(-)-Epigallocatechin	CBLnb, RGLnb.	55
8	65.2	$C_{15}H_{10}O_8$	319.0448	$[M + H]^+$	153 $[M – C_8H_5O_4]^+$, 165 $[M – C_{11}H_5O]^+$, 217 $[M – C_3HO_4]^+$, 273 $[M – CHO_2]^+$.	Myricetin	CBLnb, CSLnb, RGLnb.	52
9	31.7	$C_{16}H_{18}O_9$	355.1024	$[M + H]^+$	135 $[M – C_8H_{11}O_7]^+$, 145 $[M – C_{11}H_{13}O_4]^+$, 163 $[M – C_7H_{11}O_6]^+$.	Chlorogenic Acid	CSLnb, RGLea.	56
10	65.3	$C_{15}H_{10}O_9S$	367.0118	$[M + H]^+$	153 $[M – C_8H_5O_5S]^+$, 287 $[M – SO_3]^+$.	Kaempferol-3-*O*-sulfate	CRLnb, RGLea.	
11	66.0	$C_{19}H_{32}O_7$	373.2221	$[M + H]^+$	151 $[M – C_{15}H_{25}O]^+$, 175 $[M – C_{10}H_{13}O_4]^+$, 193 $[M – C_6H_{11}O_6]^+$, 211 $[M – C_6H_9O_5]^+$.	MCULE-1861643007	CRLnb, CSLnb, RGLnb, RGLea.	

12	32.4	$C_{16}H_{18}NaO_9$	377.0853	$[M + Na]^+$	163 $[M – C_6H_{11}O_6Na]^{+\cdot}$	MCULE-8174624502	CSLnb, RGLea.	
13	65.1	$C_{15}H_{10}O_{10}S$	383.0067	$[M + H]^+$	257 $[M – CHO_5S]^+$, 303 $[M – SO_3]^+$.	Quercetin-3-*O*-sulfate	CRLnb.	57
14	64.4	$C_{19}H_{32}O_8$	389.2170	$[M + H]^+$	209 $[M – C_6H_{11}O_6]^+$.	Icariside B5	CRLnb.	
15	66.0	$C_{20}H_{18}O_{11}$	435.0922	$[M + H]^+$	153 $[M – C_{13}H_{13}O_{11}]^+$, 285 $[M – C_9H_9O_2]^+$, 303$[M – C_5H_7O_4]^+$.	Quercetin-3-*O*-pentoside (Guaijaverin)	CSLnb, RGLea.	58
16	66.2	$C_{21}H_{20}O_{11}$	449.1078	$[M + H]^+$	129 $[M – C_{16}H_{15}O_7]^+$, 153 $[M – C_{14}H_{15}O_7]^+$, 287 $[M – C_6H_9O_5]^+$, 303 $[M – C_6H_9O_4]^+$.	Quercetin-3-*O*-rhamnoside (Quercitrin)	CRLnb, CSLnb, RGLea.	57,59
17	65.6	$C_{22}H_{18}O_{11}$	459.0922	$[M + H]^+$	123 $[M – C_{15}H_{11}O_5]^+$, 153 $[M – C_{15}H_{13}O_7]^+$.	(-)-Epigallocatechin gallate	RGLnb.	60
18	65.8	$C_{21}H_{20}O_{12}$	465.1028	$[M + H]^+$	145 $[M – C_{16}H_{15}O_7]^+$, 153 $[M – C_{14}H_{15}O_8]^+$, 247 $[M – C_{12}H_9O_4]^+$, 275 $[M – C_{11}H_9O_3]^+$, 285 $[M – C_{10}H_{11}O_3]^+$, 303 $[M – C_6H_9O_5]^+$.	Quercetin-3-*O*-galactoside (Hyperin)	CRLnb, CSLnb, RGLnb, RGLea.	6,59
19	65.6	$C_{21}H_{20}O_{12}$	465.1028	$[M + H]^+$	129 $[M – C_{15}H_{11}O_9]^+$, 153 $[M – C_{14}H_{15}O_8]^+$, 273 $[M – C_7H_{11}O_6]^+$, 301 $[M – C_6H_{11}O_5]^+$, 319 $[M – C_6H_9O_4]^+$.	Myricetin-3-*O*-rhamnoside (Myricitrin)	CBLnb, RGLnb.	57
20	65.7	$C_{21}H_{18}O_{13}$	479.0820	$[M + H]^+$	153 $[M – C_{14}H_{13}O_9]^+$, 159 $[M – C_{15}H_{11}O_8]^+$, 247 $[M – C_{12}H_7O_5]^+$, 273 $[M – C_7H_9O_7]^+$, 303 $[M – C_6H_7O_6]^+$.	Quercetin-3-*O*-glucuronide (Quercituron)	CBLnb, CRLnb, CSLnb, RGLea.	57,58,61
21	65.5	$C_{21}H_{20}O_{13}$	481.0977	$[M + H]^+$	303 $[M – C_6H_9O_6]^+$, 319 $[M – C_6H_9O_5]^+$.	Myricetin-3-*O*-galactoside	CSLnb, RGLnb.	61
22	65.5	$C_{21}H_{18}O_{14}$	495.0769	$[M + H]^+$	153 $[M – C_{14}H_{13}O_{10}]^+$, 159 $[M – C_{15}H_{11}O_9]^+$, 301 $[M – C_6H_9O_7]^+$, 319 $[M – C_6H_7O_6]^+$.	Myricetin-3-*O*-glucuronide	CBLnb.	57

23	65.5	$C_{30}H_{24}O_{12}$	577.1341	$[M + H]^+$	139 $[M – C_{27}H_{17}O_6]^+$, 425 $[M – C_{12}H_7]^+$	Proanthocyanidin A1	CSLnb, RGLea.	6
24	40.1	$C_{30}H_{26}O_{12}$	578.1424	$[M + H]^+$	139 $[M – C_{27}H_{19}O_6]^+$, 151 $[M – C_{26}H_{19}O_6]^+$, 257 $[M – C_{16}H_{17}O_7]^+$, 275 $[M – C_{20}H_{15}O_3]^+$, 287 $[M – C_{15}H_{15}O_6]^+$.	Procyanidin B2	CBLnb, CRLnb, CSLnb, RGLnb, RGLea.	62
25	64.6	$C_{27}H_{30}O_{15}$	595.1657	$[M + H]^+$	385 $[M – C_{15}H_{13}O_{11}]^+$, 559 $[M –H_3O_2]^+$.	MCULE-8196692160	CSLnb, RGLea.	
26	59.6	$C_{30}H_{32}NO_{12}$	598.1918	$[2M + NH_4]+$	123 $[M – C_7H_{25}NO_{10}]^+$, 139 $[M – C_{27}H_{25}NO_6]^+$, 291 $[M – C_{15}H_{17}NO_6]^+$.	(-)-Epicatechin	CSLnb.	
27	65.9	$C_{27}H_{30}O_{16}$	611.1607	$[M + H]^+$	153 $[M – C_{20}H_{25}O_{12}]^+$, 285 $[M – C_{16}H_{21}O_7]^+$, 303 $[M – C_{12}H_{19}O_9]^+$.	Quercetin-3-*O*-rutinoside (Rutin)	CSLnb.	57,58
28	65.6	$C_{28}H_{24}O_{16}$	617.1137	$[M + H]^+$	153 $[M – C_{21}H_{19}O_{12}]^+$, 303 $[M – C_{13}H_{13}O_9]^+$.	Quercetin 3-(2-galloylglucoside)	CRLnb, RGLnb.	57
29	65.2	$C_{27}H_{30}O_{17}$	627.1556	$[M + H]^+$	303 $[M – C_{12}H_{19}O_{10}]^+$.	Quercetin 3,4'-diglucoside	CRLnb, CSLnb.	57

277

Among the metabolites identified for these species of Connaraceae, several are implicated as promising molecules useful in the control of diabetes complications mediated by protein glycation and by the imbalance of redox metabolism. Derivatives of catechins [63] quercetin, myricetin and apigenin [13,18,63] have already had their anti-AGEs effects demonstrated. Many of the compounds identified for these species of connaraceae have already had their antioxidant effects reported by other authors [48,64–67]. Therefore, considering that in diabetes, hyperglycemia results in an increase in the production of free radicals by a mechanism that involves the oxidation of glucose followed by the glycation of proteins [68] and that the involvement of mitochondrial processes in the exacerbation of stress oxidative in response to hyperglycemia is implicated with the complications of this disease [69] the search for new therapeutic alternatives to reduce these complications is highlighted.In this context among the species *R. glazioui* has the broadest list of specialized metabolites potentially useful in the treatment of complications associated with diabetes including the compounds apigenin, kaempferol, quercetin, myricetin, chlorogenic acid and others, although the potential of other species cannot be overlooked. Plant therapies with their multiple active metabolites may in the future offer benefits in controlling diabetes complications and still have reduced toxicity [70]. Reviewing the literature, it is possible to infer that to date no antidiabetic drug has a reducing effect on protein glycation, so this is an alternative that still needs to be explored and, in this context, Connaraceae has a high potential.

Experimental Section

Plant material

The plants access was registered at the Brazilian National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen – www.sisgen.com.br).

The Table 4 shown details about the plants. These were individually dehydrated in a greenhouse with dry air flow at a controlled temperature of 35°C for 7 days, and subsequently, it`s were ground in a knife mill, *Willye* Model TE 650 Tecnal®.

Table 4. Plant material of Brazilian species (Connaraceae) used in this work.

Species	Acronyms*	Location	Biome	Coordinates	Voucher (Herbarium	SisGen
C. blanchetii	CBL	Ilhéus, BA.	Atlantic Forest	14°55′56″ S, 39°1′32″ W	C. Toledo (ESA** 143609)	A1FE9E7
C. regnellii	CRL	Piedade, SP.	Atlantic Forest	23°42′05″ S, 47°30′38″ W	C. Toledo (ESA 143607)	A7E69F6
C. suberosus	CSL	Brasília, DF.	Cerrado	16°56′54″ S, 47°51′58″ W	J. Paz (UB*** Paz 86)	A251027
R. glazioui	RGL	Conceição da Barra, ES.	Atlantic Forest	18°27′49″ S, 39°43′28″ W	C. Toledo (ESA** 143608)	A1FE9E7

* CBL = *C. blanchetii* leaves; CRL = *C. regnellii* – leaves; CSL = *C. suberosus* – leaves and RGL = *R. glazioui.* **ESA – Escola Superior de Agricultura. ***UB – Universidade de Brasília.

Extraction

Decoction – D - The decoction method was conducted following [25], with some modifications: 20 g of the obtained powder was added to water preheated at 100°C (100 mL) and maintained at a constant temperature of 100°C in heating plates for 30 min, under continual agitation. This procedure was repeated twice, and the combined supernatants were decanted and centrifuged (5 min/3000 × g/20°C), filtered (12-25 µm), solvent removed by rotative evaporation (Rotavapor® Buchi R210) and lyophilized for 24h in freeze drier (Labconco Freezone® 4.5 Plus).

Infusion – I – This method was conducted following [20], with some modifications: 20 g of the obtained powder was infused with water preheated at 70°C (100 mL) for 30 min under continual agitation at room temperature. This procedure was repeated twice, and the combined supernatants were decanted and centrifuged (5 min/3000 × g/20°C), filtered (12-25 µm),

solvent removed by rotative evaporation the recovery process were conducted according to Decoction D method.

Maceration (tincture) – M - This method was conducted according to [71], with some modifications: 20 g of the obtained powder was macerated twice in ethanol (100 mL) for 24 h under continual agitation at room temperature. After decantation and centrifugation (5 min/3000 × g/20°C), the recovered and combined supernatants were filtered (12-25 µm) and then solvent was removed by rotative evaporation.

Determination of phenolic content

Total phenolic content of CBL, CRL, CSL and RGL extracts were determined by Folin-Ciocalteu method [72] with minor modifications. Briefly, 100 µL of the extracts (1 mg/mL in distilled water) was added to 7.4 mL of distilled water and 500 µL of Folin-Ciocalteu reagent. After a 1 min of equilibration, the mixture was neutralized with 2 mL of 15% (w/w) Na_2CO_3. After 30 min of reaction, the absorbance of the mixture was measured at 750 nm in a UV/Vis spectrophotometer (Beckmann DU 530). Gallic acid (7.81–500 µg/mL) was used as a standard, and the total flavonoid content was calculated using the calibration curve for gallic acid. Amounts of phenolics were calculated from a gallic acid standard curve and expressed as µg of gallic acid equivalent/mg of dry extract.

Determination of flavonoids contents

Flavonoids contents of CBL, CRL, CSL and RGL extracts was estimated according to the method described by [72], based on aluminum chloride reaction with extract. To 1 mL of the extract (1 mg/mL in methanol) was added 4 mL of distilled water and 200 µL of 5% (w/w) $NaNO_3$. After 6 min, 200 µL of 10% (w/w) $AlCl_3$ was added, and the mixture rested for 5 min. Then, 2 mL of 10% (w/w) NaOH was added, and the total volume was brought to 10 mL with

methanol. The absorbance was measured in a UV/Vis spectrophotometer (Beckmann DU 530) after 30 min at 425 nm. Quercetin (7.81–500 μg/mL) was used as a standard, and the total flavonoid content was calculated using the calibration curve for quercetin. The absorbance of the obtained yellow complex was measured at 430 nm. Amounts of flavonoids were calculated from a quercetin standard curve and expressed as μg quercetin equivalent/mg of dry extract.

Determination of condensed tannins content

Condensed tannins contents of CBL, CRL, CSL and RGL extracts was estimated according to the method described by [73]. In this reaction the condensed tannins are converted to generate anthocyanidins [38]. Briefly, 100 μL of extract solution (250 μg/mL in methanol) were mixed with 2,5 mL of vanillin solution at 1% in methanol (w/v) and 2.5 mL of solution HCl 8% in methanol (v/v). After 15 min, then the solution was placed in a water bath previously heated to 60 °C for 10 minutes and the absorbance was measured in a UV/Vis spectrophotometer (Beckmann DU 530) at 500 nm. Catechin (62.5 – 1.000 μg/mL) was used as a standard, and the condensed tannins content was calculated using the calibration curve for catechin. Amounts of condensed tannins were calculated from a catechin standard curve and expressed as μg catechin equivalent/mg of dry extract.

Extraction and partition Maceration (tincture) – M

As previously presented here, for three cycles, 80 g of obtained powders were macerated twice in ethanol (400 mL) for 24 h under continual agitation at room temperature. After decantation and centrifugation (5 min/3000 × g/20°C), the recovered and combined supernatants were filtered (12-25 μm) and then solvent was removed by rotative evaporation (Rotavapor® Buchi R210). The dry extract was resuspended in 100 mL H_2O/ethanol 8:2 (v/v). Sequentially, the extract resuspended was degreasing with petroleum ether in separatory funnel

and extracted at room temperature with hexane, dichloromethane, ethyl acetate and n-butanol (3 x 100 mL for each solvent) [74], the figure 04 showed the process summarized.

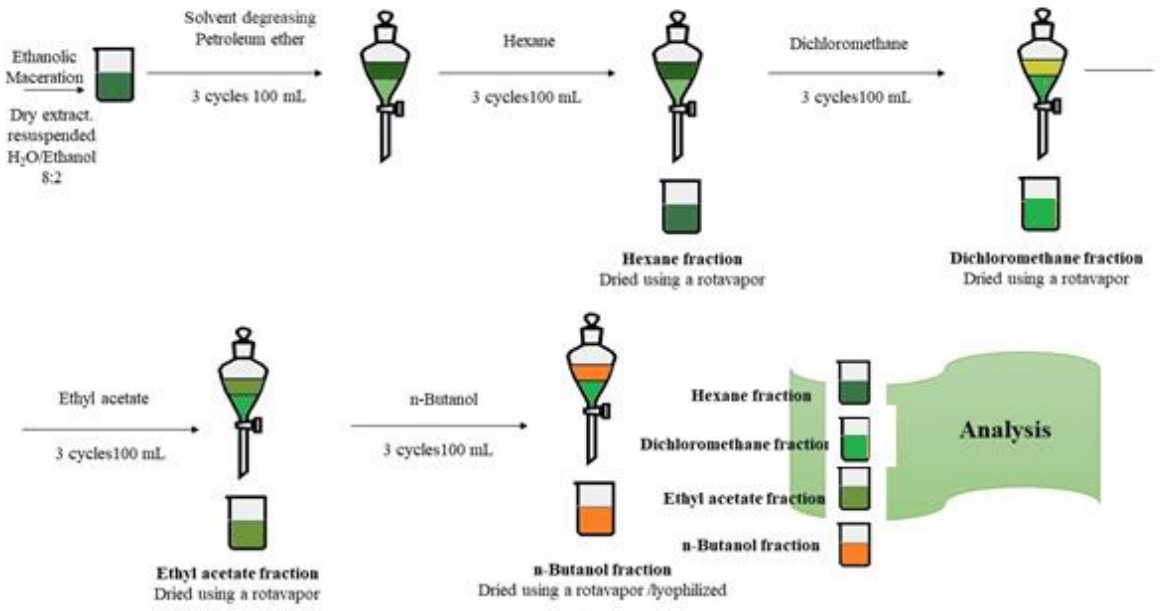

Figure 04: Schematic model for obtaining organic fractionation of the maceration extract.

Antioxidant assays

(2,2-diphenyl-1-picrylhydrazyl radical) DPPH•

Screening

A screening of the percentage of DPPH• scavenging of CBL, CRL, CSL and RGL extracts (125 µg/mL in methanol) was estimated according to the method described [75] with minor modifications. Briefly, 0.3 ml of each extract was added to 2.7 ml of DPPH• at a concentration of 40 µg/mL and the mixture was kept protected from light for 30 minutes. After the absorbance was measured in a UV/Vis spectrophotometer (Beckmann DU 530) at 515 nm. A mixture of methanol (2.7 mL) and methanolic extract solution (0.3 mL) was used as blank.

The negative control was a solution of methanol (0.3 mL) and DPPH• (2.7 mL). Tests were performed in triplicate, and DPPH• scavenging % (SC%) activity was calculated as follows: $SC\% = [(A_{Neg.control} - A_{sample})/A_{Neg.control}] \times 100$. Where, $A_{Neg.control}$ and A_{sample} are the average absorbance values of the negative control and samples, respectively.

Determination of scavenging concentration

The scavenging concentration (SC_{50}) of the ethyl acetate and n-bunanolic fractions was determined using serial dilutions of the dry extract (15.6 to 62.5 µg/mL) following the same method previously presented.

Ferric reducing antioxidant power (FRAP) Assay

The FRAP of the ethyl acetate and n-bunanolic fractions was determined assay was performed according to the method used by [76], with slight modifications. Stock solutions were composed from: acetate buffer 300 mM, pH 3.6 (3.1 g of sodium acetate, 16 ml of glacial acetic acid with the volume completed up to 1L with deionized water); 2, 4, 6-tripyridyl-s-triazine (TPTZ) solution 10 mM (3.12 g of TPTZ dissolved in a 40 mM HCl aqueous solution with the volume made up to 1L) ; Ferric Chloride Solution ($FeCl_3 \cdot 6H_2O$) 20 mM (5.4 g of $FeCl_3 \cdot 6H_2O$ dissolved in deionized water up to 1 L); The FRAP solution solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \cdot 6H_2O$ solution and then warmed at 37 °C before using. Connaraceae ethanolic extracts 16,2 to 250 µg/mL (90µL), deionized water (270 µL) were allowed to react with 2850 µL of the FRAP solution for 30 min in the dark condition. The colored product [ferrous tripyridyltriazine complex] was then measured at 595 nm in a UV/Vis spectrophotometer (Beckmann DU 530). The standard curve

was linear between 500 to 2.000 μM using ferrous sulphate ($FeSO_4 \bullet 7H_2O$) was performed according [77]. The results were expressed in ug.ext.dry/mL eq. 1.000 mM ($FeSO_4 \bullet 7H_2O$).

Inhibitory effect in Advanced Glycation Endproducts (AGEs)

Bovine serum albumin and glyoxal model (BSA/GO)

The method of measuring anti-AGE activity *via* the oxidative pathway [78], was prepared in accordance with the proposed modifications [43]. The dry extracts were prepared in DMSO (100 ug mL^{-1}). The glyoxal (30 mM) and BSA (bovine serum albumin) (10 mg mL^{-1}) solution was prepared in phosphate buffer (0.2 M, pH 7.4) containing 3.0 mM sodium azide as an antimicrobial agent. The reactions were performed with 300.0 μL of the total reaction mixture composed by [BSA (135.0 μL), glyoxal (135.0 μL) and dimethyl sulfoxide or sample (30.0 μL)], and incubated at 37 °C. After 48 h of incubation, the sample was analyzed for fluorescence intensity using a microplate reader (excitation at 330 nm and emission at 420 nm) (DTX 800, Beckman Coulter, CA, USA). DMSO was used as the negative control, and quercetin (100.0 μg/mL) was used as the standard. The experiment was performed in triplicate. The percentage of inhibition was calculated using the equation:

[% inhibition = 100 − (A_2 sample – A_1 sample/A_2 control − A_1 control) × 100], where A_1 is the fluorescence of the initial reading and A_2 is the fluorescence of the final reading.

For all extracts at 100 μg/mL whose inhibition percentage was greater than 50%, the respective IC_{50} were determined using serial dilutions of the dry extract (10 – 100.0 μg/mL) in DMSO.

Bovine serum albumin and fructose model (BSA/fructose)

Anti-AGE activity, measured using the non-oxidative pathway method, was determined according to the method described by [78] with some modifications [43]. Utilizing the same methodology as described for Bovine serum albumin and glyoxal model (BSA/GO), the

incubation time was set at 72 h and used fructose (0.10 mM) instead of glyoxal. Aminoguanidine was used as the standard. The assay was performed in triplicate. The IC_{50} was determined using serial dilutions of the dry extract (6.0 – 100.0 μg/mL) in DMSO.

Chemical composition

LC-MS/MS analysis

The LC-MS analysis was performed by [9], in Shimadzu 20A HPLC system with binary solvent delivery, degas system, auto sampler and SPD-20A UV-Visible detector (dual channel ƛ254 and 320 nm). Separation method was performed with an ODS C_{18} analytical column (4.6 × 250 mm), with particles of 5 μm. The mobile phase was in gradient mode: A - water/formic acid 0.1% v/v; B - and methanol/formic acid 0.1% (v:v), which were eluted at 1 mL/min as follows: 13.8% of B at 0-45 min; 28% of B at 45-60 min; 100% of B at 60-80 min and finally with 13.8% of B at 80-82 min. Mass spectrometric analysis was performed using Bruker® MicroTof-QII spectrometer with Electrospray Ionization source (ESI) operated in positive ionization mode. ESI source was operated at 200°C with an ionization voltage of 35-40 *eV* and sheath gas flow rate of 8 L/min. The analysis was performed at *m/z* range of 100-1200 and a normalized collision energy of 10 *eV* at 15000 resolution (FWHM) was used for the survey scans.

Data analysis - molecular network

A molecular network was created using the online workflow (https://ccms-ucsd.github.io/GNPSDocumentation/) on the GNPS website (http://gnps.ucsd.edu)[79]. The spectra were window filtered by choosing only top 6 fragment ions in the +/- 50 Da window throughout the spectrum. The precursor ion mass tolerance was set to 0.02 Da, with a MS/MS fragment ion tolerance of 0.02 Da [80]. A network was then created in which edges were filtered to have a cosine score above 0.7 and more than 6 matched peaks. In addition, edges between

two nodes were only kept in the network if each of the nodes appeared in each other's respective top 10 most similar nodes. Finally, the maximum size of a molecular family was set to 100, and the lowest scoring edges were removed from molecular families until the molecular family size was below this threshold. The spectra in the network were then searched against GNPS spectral libraries. The library spectra were filtered in the same manner as the input data. All matches between network spectra and library spectra were required to have a score above 0.7 and at least 6 matched peaks. The results were downloaded and exported to be visualized on Cytoscape 3.8.2 software [https://cytoscape.org].

Complementary analysis – CFM-ID and ChemCalc plataforms

To complement and confirm the identification made by the GNPS platform, in addition to the retention times of the metabolites present in the extracts, tool spectral prediction was used (https://cfmid.wishartlab.com). This tool provides low energy/10 V, medium energy/20 V and high energy/40 V MS/MS spectra for a necessary input structure in the Simplified Molecular Input Line Entry System (SMILES) format. Spectra of compounds are produced using combinatorial fragmentation [45,46]. The SMILES of the compounds were obtained from the website (https://pubchem.ncbi.nlm.nih.gov/), and the data were then submitted to the work tool flows in the following parameters: spectra type: ESI; ion mode: Positive; adduct type: $[M+H]^+$. Spectra peaks and possible matching fragments for the compounds were evaluated in 40 V, a similar energy to that used in the LC/MS analysis. Additionally, all matching fragments had their chemical formulas searched in the MF Finder tool on the ChemCalc platform [47].

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Capítulo 4

Metodologia e resultados referentes aos objetivos específicos 7 e 8.

Materiais e métodos

Determinação das atividades de inibição enzimática e determinação da composição química.

As avaliações da atividade inibitória de α-amilase e α-glucosidase foram conduzidas de acordo com os métodos a seguir apresentados para as frações n-butanólicas das folhas de *C. blanchetii*, *C. nodosus*, *C. regnellii*, *C. suberosus*, *R. glazioui* e *R. induta*, fração acetato de etila das folhas de *R. glazioui* e para as frações n -butanólicas das cascas de *C. regnellii*, *C. suberosus* e *R. cuspidata.*

A atividade inibitória da α-amilase

A atividade inibitória da α-amilase seguiu o protocolo colorimétrico estabelecido por Oliveira e cols (2021). Inicialmente 100,0 μL da enzima α-amilase e 30 μL dos extratos secos (1,0 mg mL^{-1} previamente dissolvidos em dimetil sulfóxido (DMSO)) foram incubados por 5 min a 37 ° C, então 170,0 μL do substrato (2-cloro-4-nitrofenil α-D-maltotriosídeo) (CNPG3) foram adicionados e a reação foi imediatamente medida a 405 nm (valor A1) no leitor de microplacas (DTX 800, Beckman Coulter, CA, EUA). As placas foram incubadas a 37 ° C por 40 min e posteriormente, foi realizada a leitura da absorbância final da amostra (A2).

Os compostos acarbose e naringenina foram utilizados como padrões. DMSO foi usado como controle em branco (controle negativo). A porcentagem de inibição foi calculada usando a equação:

$$\text{Percentual de inibição} = 100 - (A_2 - A_1) \times 100$$

A atividade inibitória da α-glucosidase

A atividade inibitória da α-glucosidase foi determinada de acordo com (OLIVEIRA *et al.*, 2021) Esse experimento baseia-se na liberação de 4-nitrofenol pela α-glucosidase partir do 4-nitrofenil α-D-glucopiranosídeo (4-NPGP). Inicialmente, 170 μL da enzima α-glucosidase murina (3,0 mg mL^{-1} em tampão fosfato 10 mM, pH 6,9) foram adicionado a 30,0 μL dos extratos secos (1,0 mg mL^{-1} previamente dissolvidos em DMSO). Após 2 min de incubação a 37 ° C, a absorbância inicial (A1) da amostra foi lida, então a reação foi iniciada pela adição de 100 μL do reagente de cor 4-NPGP (5,0 mg mL^{-1} em tampão fosfato 10 mM, pH 6,9). Esta reação foi mantida por mais de 2 h a 37 ° C, então a absorbância final (A2) foi lida.

Acarbose (100 μg mL^{-1}) foi utilizada como padrão positivo e DMSO foi usado como controle em branco (controle negativo). A porcentagem de inibição foi calculada usando a equação:

Percentual de inibição = $100 - (A_2 - A_1) \times 100$

Determinação da composição química

O estudo da composição química foi conduzido de acordo com nosso prévio artigo (PAIM *et al.*, 2021).

Avaliação de toxicidade e reversão da citotoxicidade induzida pela hiperglicemia em células endoteliais EA.hy926

Com base nos resultados obtidos optamos por avaliar as frações n-butanólicas das folhas de *C. suberosus* e *R. glazioui,* fração acetato de etila das folhas de *R. glazioui,* e fração n - butanólica das cascas de *R. cuspidata.*

Preparação dos Extratos

As frações secas das 4 amostras, a) *C. suberosus* - fração n-butanol, b) *R. glazioui* - fração - acetato de etila, c) *R. glazioui* - fração - n-butanol e d) *R. cuspidata* - fração n-butanol foram extraídas com água destilada a 85ºC na concentração de 1% (p/v). Agitadas manualmente e mantidas em banho-maria por 15min, sob a mesma temperatura. Após, foram filtradas em bomba a vácuo e centrifugadas a 3.000 rpm por 20min. O sobrenadante foi coletado e mantido congelado a -20°C para posteriores testes.

Desenho Experimental

A linhagem endotelial EA.hy926 (ATCC® CRL-2922), foi cultivada em meio *Dulbecco's Modified Eagle Medium* (DMEM) suplementado com 10% de soro fetal bovino (SFB) e 1% de antibiótico (penicilina/estreptomicina). As culturas foram mantidas em estufa com temperatura e umidade controladas a 37ºC e 5% de CO_2, respectivamente. Para os tratamentos, as células foram lavadas com tampão salino e soltas do recipiente com solução

de tripsina 0,15%. Em seguida, inoculadas em placas de 96 poços contendo meio DMEM completo, mantidas em estufa para aderência por 24h. Após, o meio de cultura foi trocado e adicionado de diferentes concentrações dos extratos (10, 20, 50, 100μg/mL) sem e com adição de glicose 35mM (HIGH), sob privação sérica. Ambos permanecendo por mais 24h.

Determinação da Viabilidade Celular

A viabilidade celular e citotoxicidade foi mensurada através do ensaio com brometo de 3-[4,5-dimetiltiazol 2-il]-2,5-difeniltetrazólio (MTT) (MOSMANN, 1983). As células tratadas foram lavadas com tampão salino e então adicionadas de 0,1mL de meio DMEM contendo o reagente MTT, incubadas por 3h. Posteriormente, a solução foi retirada e a placa deixada secar a temperatura ambiente por 48h. Os cristais formados foram dissolvidos em 0,3mL de DMSO para a realização da contagem espectrofotométrica de mitocôndrias viáveis em leitor de microplacas VICTOR modelo X3 a 517nm. Os resultados foram expressos em % do controle. Para a avaliação morfológica da linhagem, utilizou-se microscópio invertido OPTIPHASE (modelo 38469) e as imagens capturadas pelo programa TSVIEW 7.3.1.7.

Análise Estatística

As análises foram realizadas em duplicata e os resultados expressos em média e desvio padrão. Os dados foram verificados quanto à normalidade por *Kolmogorov-Smirnov* e os resultados submetidos à análise de variância (ANOVA) seguida de Teste *post hoc* de Tukey. Significância estatística de $p<0,05$, usando software SPSS versão 22.0 (SPSS, Chicago, IL).

Resultados e discussão

A busca por compostos de origem vegetal com capacidade de inibir enzimas relacionadas como metabolismo de carboidratos e consequente redução da glicemia está reportada na literatura para inúmeras espécies vegetais (BAINS; GUGLIUCCI, 2017; BROHOLM *et al.*, 2019; KWON; APOSTOLIDIS; SHETTY, 2008; STOJKOVIC *et al.*, 2019; WANG; DU; SONG, 2010; YIN *et al.*, 2018). Entre as enzimas a α-amilase, figura 19, poderia ser um potencial alvo de inibição, pois, através dela os carboidratos como o amido, por

exemplo, sofrem hidrólise nas ligações α1,4 e consequentemente liberam seus monômeros (glicose) os quais poderão ser então absorvidos pela corrente sanguínea.

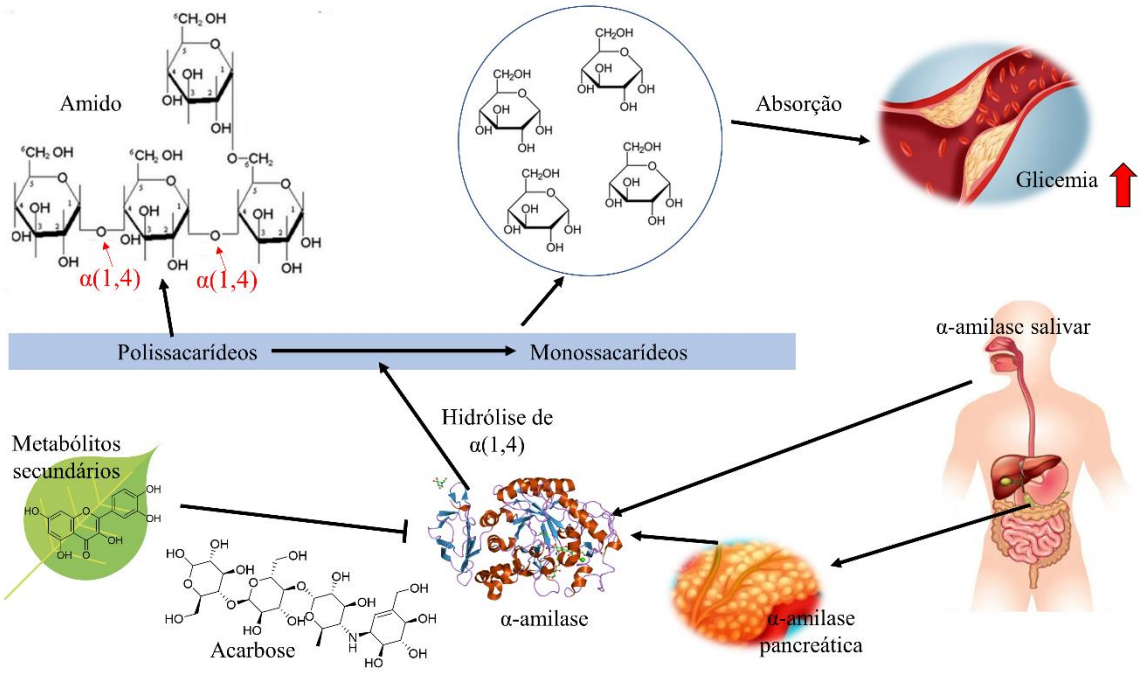

Figura 19: O envolvimento da α-amilase no metabolismo dos carboidratos.
Fonte o autor.

A α-glucosidase é outra enzima relacionada ao metabolismo de carboidratos, e do mesmo modo que para a α-amilase, sua inibição está implicada com a redução na absorção de glicose (AHMED *et al.*, 2019). A α-glucosidase encontra-se associada as células do intestino responsáveis pela absorção da nutrientes (UMAMAHESWARI; SANGEETHA, 2019). Nas bordas em escova dos enterócitos a α-glucosidase atua sobre oligossacarídeos não absorvíveis e através de reações de hidrólise libera os açúcares na forma de monossacarídeos os quais então podem ser aborvidos (PATIL *et al.*, 2015). Portanto, compostos químicos que atuem como inibidores de a α-amilase e α-glucosidase podem trazer benefícios aos pacientes portadores de DM.

A inibição da absorção de carboidratos no intestino delgado pode ser alcançada por fármacos como a acarbose a qual atua competitivamente pela enzima α-glucosidase (AKMAL; WADHWA, 2021). Em experimentos *in vitro* a acarbose tem sido usada como composto padrão positivo para averiguação de atividades inibitórias α-glucosidase (AHMED *et al.*, 2019; ANDRADE-CETTO; BECERRA-JIMÉNEZ; CÁRDENAS-VÁZQUEZ, 2008; BROHOLM *et*

al., 2019; CAI *et al.*, 2020; ZHANG *et al.*, 2019), esse composto também tem sido empregado para testes como α-amilase (OLIVEIRA *et al.*, 2021; SUBRAMANIAN; ASMAWI; SADIKUN, 2008).

Portanto, a busca compostos químicos a partir de fontes naturais os quais possam promover inibição da α-amilase e α-glucosidase pode ser considerada uma das estratégias para o controle da hiperglicemia associada ao DM. Neste trabalho as frações n-butanólicas de todas as sete espécies e a fração acetato de etila obtida de *R. glazioui* foram testadas na concentração de 1,0 mg mL^{-1} quanto ao potencial inibitório das enzimas α-amilase e α-glucosidase (tabela 4).

Tabela 4: Percentual de inibição dos extratos (1,0 mg mL^{-1} / DMSO) frente as enzimas α-amilase e α-glucosidase.

Amostras	Percentual de inibição α-amilase Média± Desvpad	Percentual de inibição α-glucosidase Média± Desvpad
Folhas (n-butanol)		
C. blanchetii	5,5±0,9	3,8±1,1
C. nodosus	6,0±1,5	7,0±1,2
C. regnellii	2,9±0,9	10,4±0,5
C. suberosus	29,0±0,7	7,6±0,6
R. glazioui	10,9±0,8	7,1±1,8
R. induta	46,6±1,2	5,1±1,4
Folhas (acetato de etila)		
R. glazioui	38,1±1,1	2,7±1,3
Cascas (n-butanol)		
C. regnellii	4,2±0,6	7,1±0,7
C. suberosus	27,9±1,2	7,9±0,2
R. cuspidata	5,3±1,3	4,7±0,6
Padrão 100 μg mL^{-1}		
Acarbose	79,5±2,7	59,6±3,2

Frente a atividade inibitória de α-amilase para as frações n-butanólicas das folhas de Connaraceae as espécies *R. induta* e *C. suberosus* apresentaram os maiores percentuais inibitórios com 46,6±1,2, 29,0±0,7. Na avaliação a partir da fração acetato de etila de *R. glazioui* – folhas o percentual de inibição foi de 38,1±1,1. Por fim, em relação as cascas a espécie *C. suberosus* apresentou o melhor perceutal inibitório com 27,9±1,2. Avaliando os resultados é possível observar que todas as espécies de Connaraceae, nas condições experimentais ensaiadas, apresentaram algum percentual de inibição de α-amilase, embora mostram-se menos ativas do que o padrão acarbose 79,5±2,7.

Para a atividade inibitória de α-glicosidase o maior efeito foi observado para *C. regnellii* cujo percentual de inibição foi de 10,4±0,5. Frente a todas as outras amostras os percentuais de

inibição de α-glicosidase mostram-se menores do que 10% e são inferiores ao resultado apresentado pelo padrão acarbose 59,6±3,2.

A composição química dessas espécies foi estudada através da construção de redes moleculares (PAIM *et al.*, 2021). Na figura 20 estão representados os clusters referentes aos flavonoides e outros compostos das frações n-butanólicas obtidas do macerado das folhas de *C. blanchetii*, *C. nodosus*, *C. regnellii*, *C. suberosus*, *R. glazioui* e *R. induta*, enquanto que na figura 21 está representado o cluster referente às procianidinas e compostos relacionados para mesmas espécies. A figura 22, apresenta a composição química da fração acetato de etila das folhas de *R. glazioui*. Finalmente, os compostos relacionados as frações n -butanólicas das cascas de *C. regnellii*, *C. suberosus* e *R. cuspidata* são mostrados na figura 23.

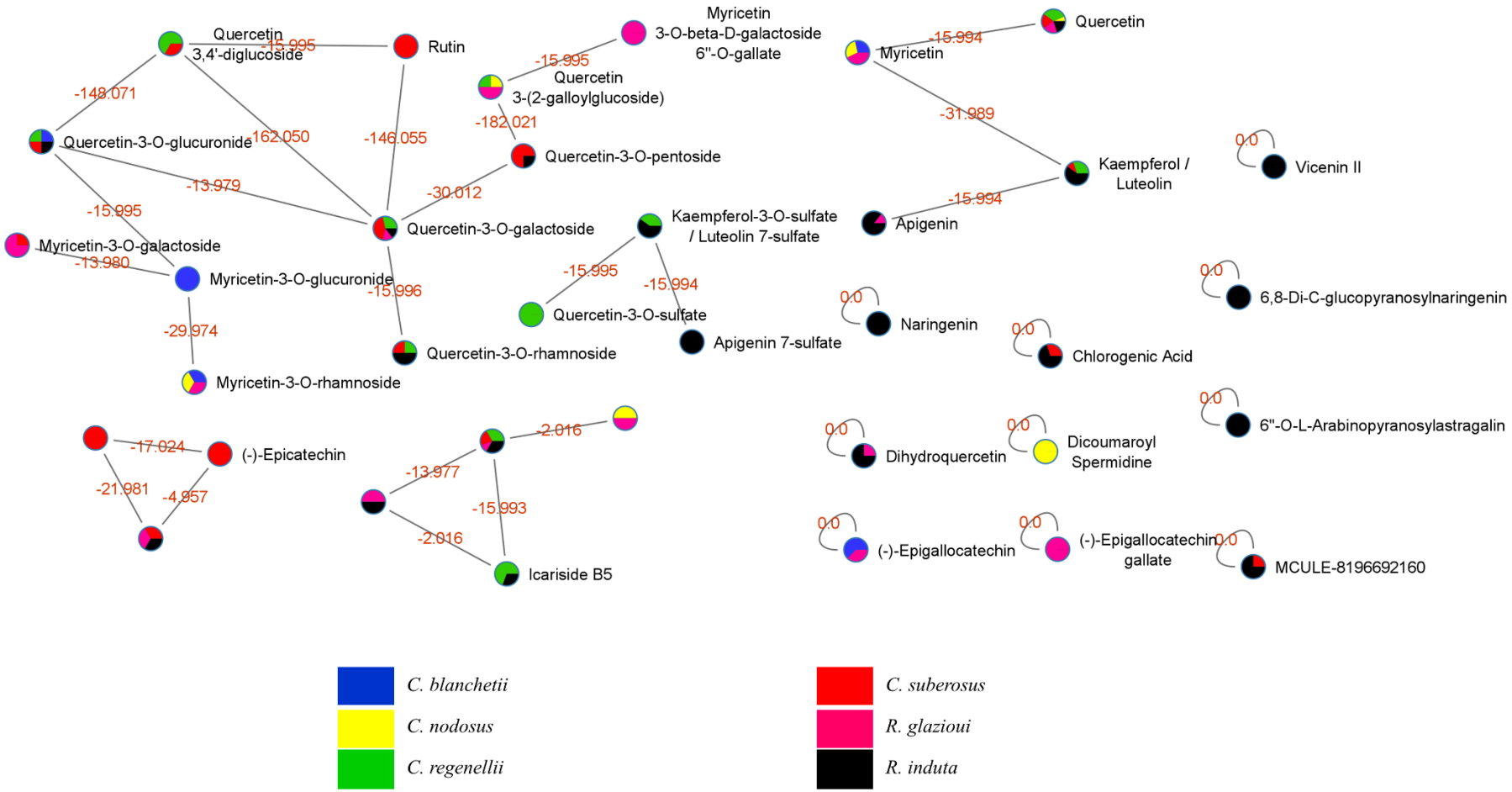

Figura 20: Flavonoides e outros compostos químicos identificados nas frações n-butanol - folhas em Connaraceae.

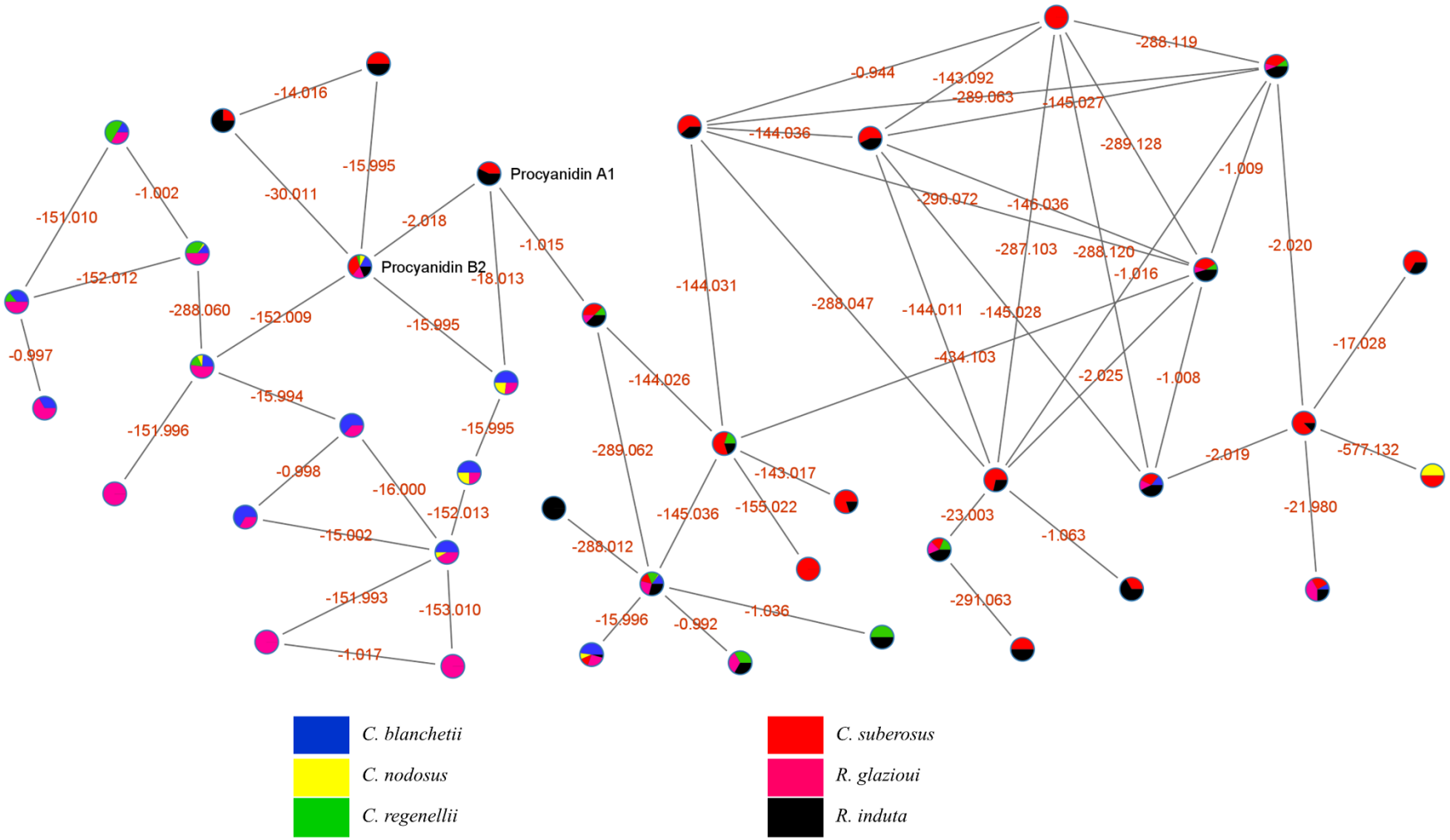

Figura 21: Cluster referente procianidinas e compostos relacionados identificadas nas frações n-butanol - folhas em Connaraceae.

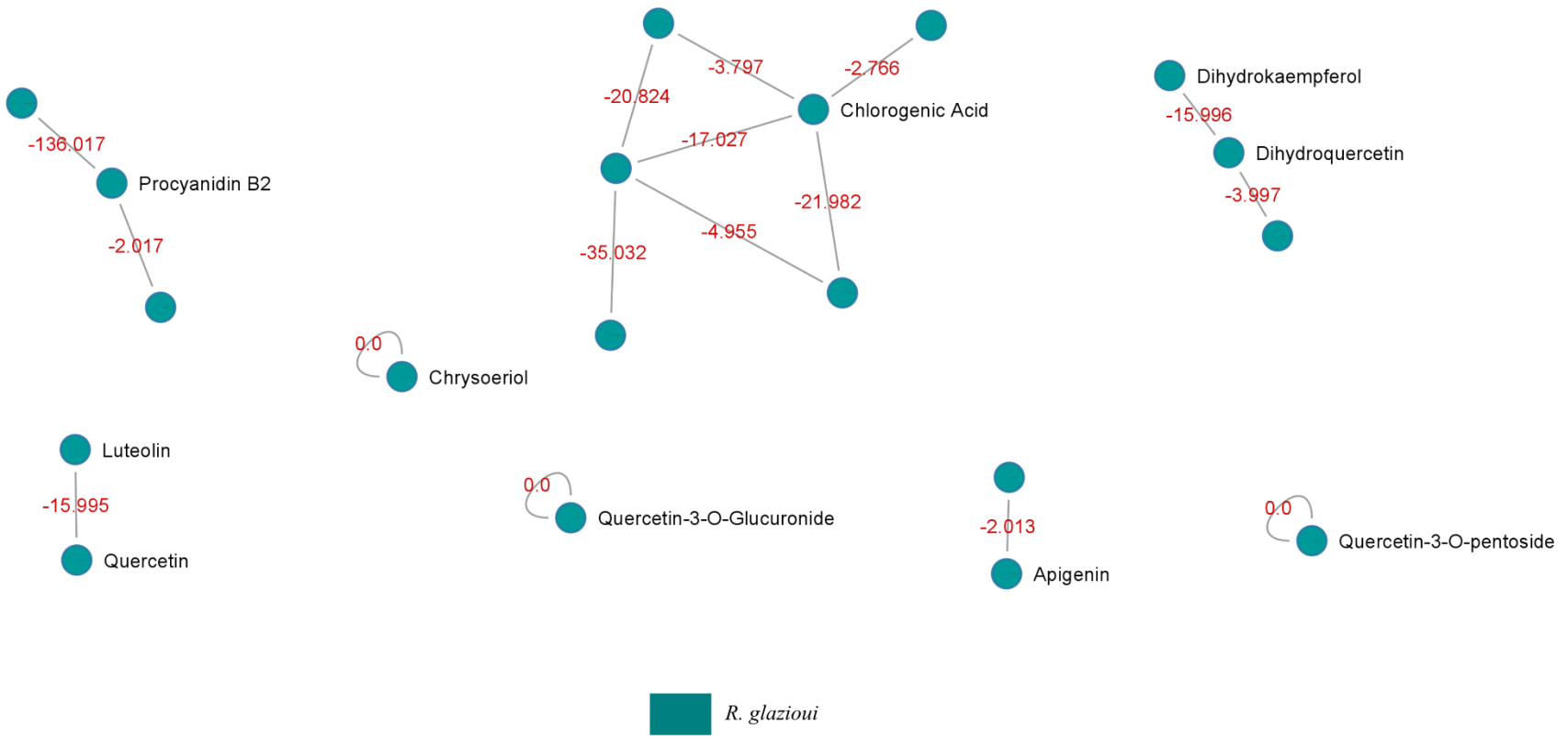

Figura 22: Flavonoides e outros compostos químicos identificados nas folhas de *Rourea glazioui* -fração acetato de etila.

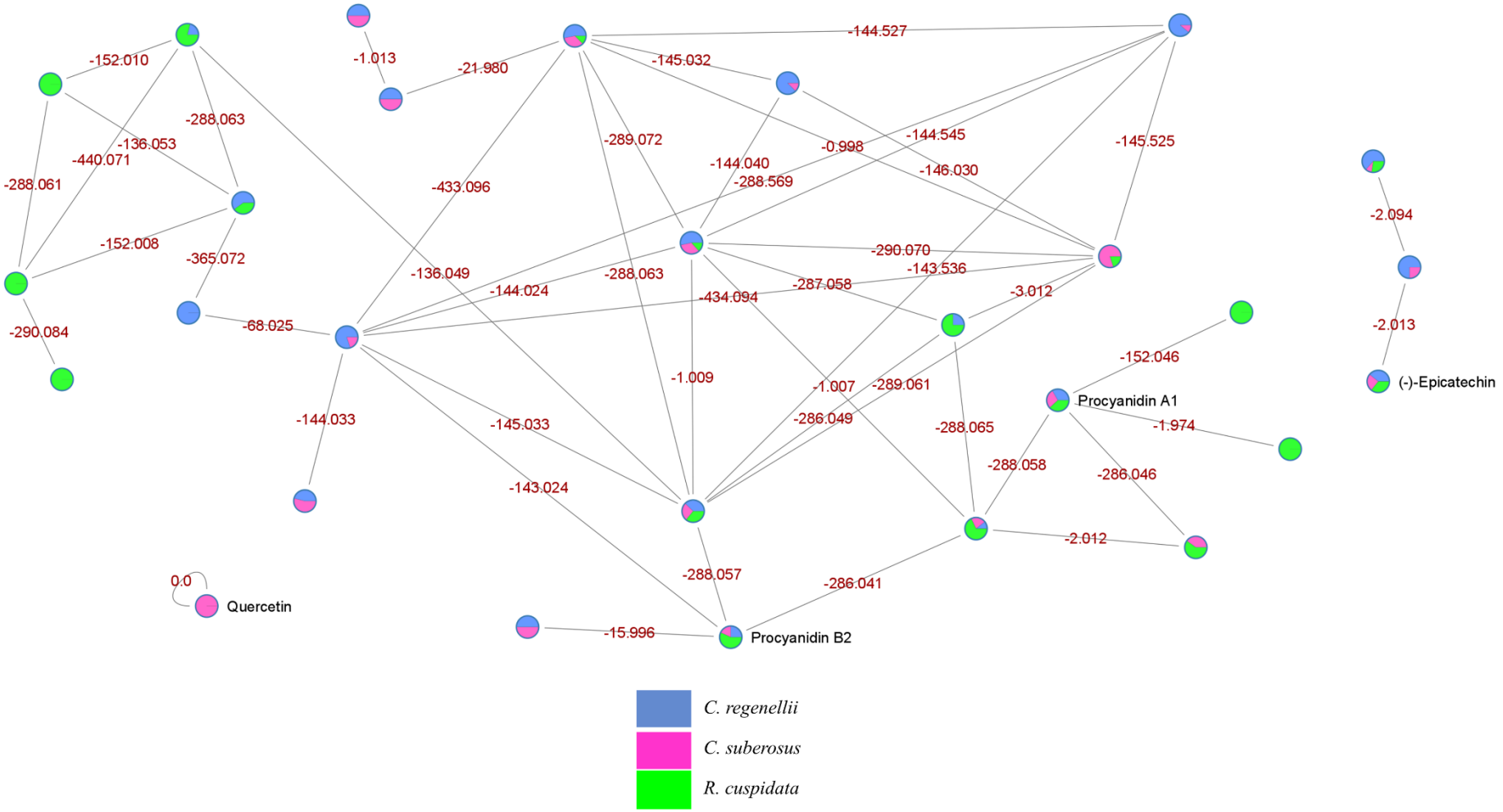

Figura 23: Flavonoides e procianidinas identificadas nas cascas de de *C. regnellii*, *C. suberosus* e *R. cuspidata* fração n-butanol.

Entre os principais metabólitos identificados entre as frações n-butanólicas estão os flavonóis camferol em *C. regnellii* e *C. suberosus*, quercetina em *C. nodosus, C. regnellii, C. suberosus, R. glazioui* e *R. induta* e miricetina para *C. blanchetii, C. nodosus* e *R. glazioui* e as flavonas apigenina *R. glazioui* e *R. induta* e luteolina em *R. induta*. Os derivados de quercetina e miricetina formam um cluster com 11 heterosídeos, muitos já destes metabólitos já relatados para o gênero *Connarus* nosso prévio trabalho (PAIM *et al.*, 2021). Em adição, nas frações n-butanólicas, as procianidinas A1 em *C. suberosus* e *R. induta* e B2 para todas as espécies foram identificadas. Na fração acetato de etila das folhas de *R. glazioui* os compostos luteolina, crisoeirol, dihidrocamferol, ácido clorogênico, quercetina-3-*O*-pentosídeo e quercetina-3-*O*-gluconorídeo foram identificados em adição a outros compostos já relatados para a fração n-butanol dessa espécie. Finalmente, na avaliação dos metabólitos associados as cascas, as procianidinas A1 e B2 e (-)-epicatequina aparecem onipresentes para *C. regnellii*, *C. suberosus* e *R. cuspidata*, enquanto que a quercetina pode ser identificada apenas para *C. suberosus.*

A associação entre as atividades inibitórias observadas nos ensaios com α-amilase e α-glucosidase e os compostos identificados nas frações n-butanol e acetato de etila das folhas em Connaraceae permite inferir que o metabólito quercetina-3-*O*-gluconorídeo encontra-se implicado com parte dos efeitos observados. Isto porque quercetina-3-*O*-gluconorídeo já está relatado com a inibição das enzimas α-amilase e α-glucosidase (AHMED *et al.*, 2019). Em outro estudo, que avaliou a atividade inibitória das enzimas α-amilase e α-glucosidase, os compostos apigenina, camferol, quercetina, miricetina, quercetina-3-*O*-pentosídeo (guaijaverina), quercetina-3-*O*-galactosídeo (hiperina) demonstraram capacidade similar ao observado para o padrão acarbose (WANG; DU; SONG, 2010). Portanto, considerando a composição química das frações n-butanol das folhas de *R. induta* é possível inferir que os compostos apigenina, quercetina, guaijaverina, hiperina e quercetina-3-*O* gluconorídeo atuam provavelmente sinergicamente e são os responsáveis pela inibição enzimática. Em *C. suberosus* camferol, quercetina, guaijaverina, hiperina e quercetina-3-*O* gluconorídeo estão associados ao efeito observado.

Para a fração acetato de etila de *R. glazioui* - folhas as presenças de quercetina-3-*O*-pentosídeo e quercetina-3-*O*-gluconorídeo podem ter sido decisivas para que o efeito inibitório da α-amilase ser maior do que o observado para a fração n-butanólica.

Nas atividades observadas para as cascas de *C. regnellii*, *C. suberosus* e *R. cuspidata* o maior percentual de inibição de α-amilase produzido por *C. suberosus* provavelmente está associado a presença de quercetina entre os metabólitos identificados para essa espécie.

A partir de todos os resultados obtidos nesta e nas outras fases deste trabalho, optamos por conduzir uma avaliação da possível toxicidade dos extratos de *C. suberosus* (n-butanol), *R. cuspidata* (n-butanol) e *R. glazioui* (acetato de etila e n-butanol) utilizando células endoteliais da linhagem EA.hy926. A escolha por essas espécies deu-se em razão de que *R. cuspidata* já apresentou efeito antidiabético, numa abordagem *in vivo*, em estudo anteriormente conduzido por nosso grupo (LAIKOWSKI *et al.*, 2017), enquanto que *C. suberosus* e *R. glazioui* apresentaram bom perfil antioxidante e um extenso rol de metabólitos potencialmente úteis ao controle do diabetes e suas complicações. A exclusão de *R. induta* deste rol de espécies deu-se em razão de reserva de patente em favor de outro grupo de pesquisa (DALLARMI *et al.*, 2012).

A figura 24 (a-d) apresenta os resultados dos ensaios de toxicidade frente as células EA.hy926 avaliado pelo percentual de viabilidade celular através do ensaio com MTT.

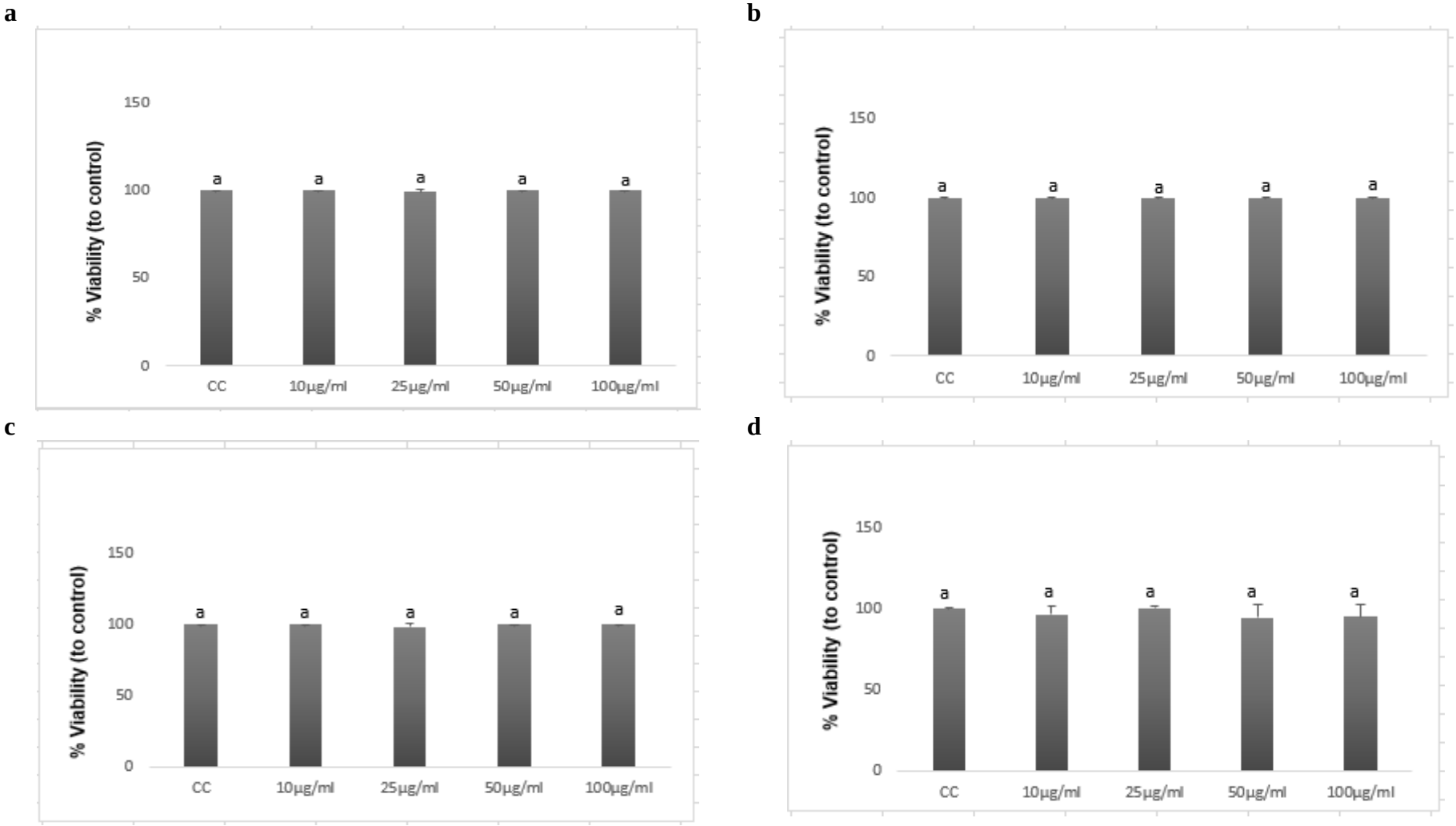

Figura 24: Avaliação de toxicidade em células endoteliais EA.hy926 meio DMEM completo (normoglicêmico), para os extratos nas diferentes concentrações (10, 20, 50, 100 µg/mL). a) *C. suberosus* - fração n-butanol, b*) R. cuspidata* - fração n-butanol, c) *R. glazioui* - fração acetato de etila e d) *R. glazioui* - fração n-butanol.

A avaliação dos resultados obtidos para o ensaio de toxicidade mostrou que para as concentrações testadas nenhum extrato apresentou toxicidade. Os percentuais de viabilidade celular observados, mesmo para as concentrações mais altas dos extratos (100 µg/mL), para *C. suberosus* (n-butanol), *R. cuspidata* (n-butanol) e *R. glazioui* (acetato de etila e n-butanol) apresentam-se estatisticamente iguais aos encontrados para os respectivos controles. Portanto, através desse experimento, infere-se, de modo preliminar, que os extratos associados a essas espécies não apresentam toxicidade, embora, experimentos adicionais e complementares ainda precisam ser conduzidos.

A disfunção endotelial em função da exposição celular a altas concentrações de glicose está relatada na literatura com uma das principais alterações associados ao diabetes (COHEN *et al.*, 2007; LI *et al.*, 2016). As células endoteliais formam uma monocamada que reveste o lúmen dos vasos e consequentemente estão em contato direto com sangue (BONETTI; LERMAN; LERMAN, 2003). Portanto, na presença de hiperglicemia associada ao diabetes descompensado as células endoteliais encontram-se expostas à altas concentrações de glicose e consequentemente estão mais susceptíveis aos danos celulares. Conforme relatado na literatura a hiperglicemia está associada ao estresse oxidativo (ASMAT; ABAD; ISMAIL, 2016; COHEN *et al.*, 2007; MARITIM; SANDERS; WATKINS, 2003), portanto considerando o potencial antioxidante de muitos dos compostos identificados para Connaraceae (DOMITROVIĆ *et al.*, 2015; KALEGARI *et al.*, 2014b; PAIM *et al.*, 2021; PIRES *et al.*, 2017) é possível vislumbrar que vários possam ser úteis no controle das complicações do DM. Ainda neste contexto, outros trabalhos têm reportado que diferentes classes de flavonoides apresentam efeitos benéficos na prevenção e redução dos complicações cardiovasculares associadas a disfunção endotelial (YAMAGATA; YAMORI, 2020). Portanto, com vistas avaliar uma possível reversão da citotoxicidade induzida pela hiperglicemia para células endoteliais EA.hy926 expostas à altas concentrações de glicose (meio DMEM completo com adição de glicose 35Mm (HIGH)) propomos estudar o efeito protetor dos extratos de *C. suberosus* (n-butanol), *R. cuspidata* (n-butanol) e *R. glazioui* (acetato de etila e n-butanol) figura 25 (a-d).

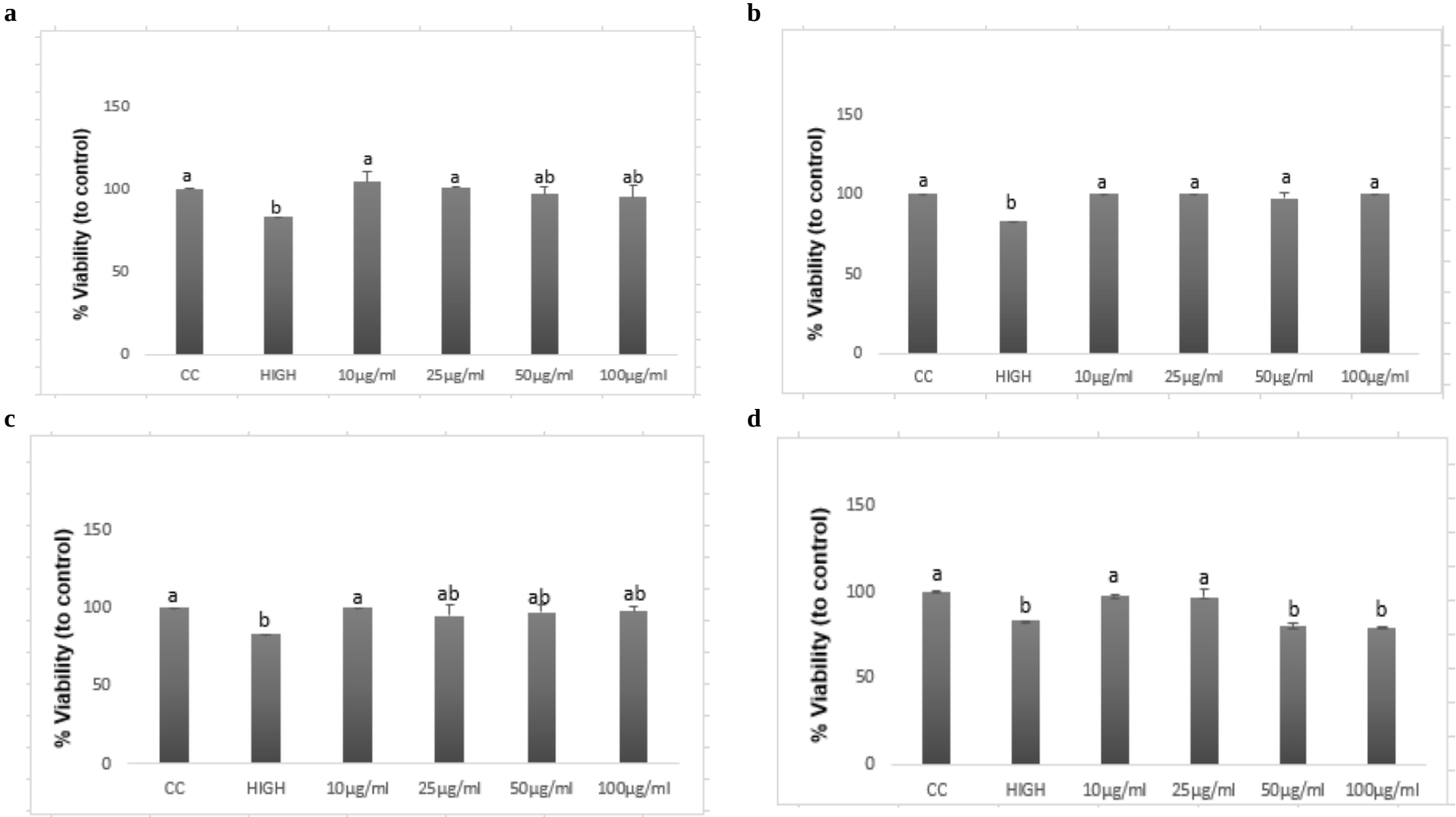

Figura 25: Avaliação de reversão de toxicidade em células endoteliais EA.hy926 meio DMEM completo (hiperglicêmico com adição de glicose 35mM), para os extratos nas diferentes concentrações (10, 20, 50, 100 µg/mL). a) *C. suberosus* - fração n-butanol, b*) R. cuspidata* - fração n-butanol, c) *R. glazioui* - fração acetato de etila e d) *R. glazioui* - fração n-butanol.

A redução do percentual de viabilidade celular observado para as células endoteliais na exposição ao meio HIGH indica que a glicose induz a danos celulares capazes de provocar a morte celular. Para *C. suberosus* (n-butanol), figura 25a, frente as concentrações de 10 e 25 µg/mL é possível observar que o percentual de viabilidade é estatisticamente igual ao observado para o meio controle sem glicose, por outro lado, é maior que o percentual de viabilidade produzido pelo meio HIGH sem qualquer adição de extrato. Portanto, o extrato *C. suberosus* (n-butanol) apresenta reversão da citotoxicidade quanto testado nas concentrações de 10 e 25 µg/mL, embora para as concentrações mais altas tal efeito não tenha sido observado. Para *R. cuspidata* (n-butanol), figura 25b, observa-se que todas as concentrações são capazes de promover reversão da citotoxicidade mediada por glicose. Para *R. glazioui* (acetato de etila) figura 25c, o efeito protetor foi observado apenas para o extrato testado na concentração de 10 µg/mL. Finalmente, para *R. glazioui* (n-butanol), figura 25d, as concentrações de 10 e 25 µg/mL mostraram efeito protetor. Assim, tomando como base os efeitos observados para todas as espécies é possível inferir que frente a maioria as concentrações de 10 e 25 µg/mL apresentam os melhores desempenhos quanto a reversão da citotoxicidade induzida pela hiperglicemia.

Assim, tomando como base a composição química de Connaraceae é possível inferir que a reversão da citotoxicidade está provavelmente associada ao efeito sinérgico dos diferentes compostos fenólicos principalmente dos flavonoides os quais reduzem os danos celulares através dos seus efeitos antioxidantes.

5 DISCUSSÃO GERAL

Na primeira etapa de construção desse trabalho, através da busca de artigos acerca de Connaraceae evidenciamos a necessidade de sistematizar todas as informações a respeito da distribuição mundial, utilização etnobotânica, fitoquímica, atividades farmacológicas e toxicologias disponíveis. Utilizando alguns critérios de buscas já referendados (MEDEIROS *et al.*, 2013), com algumas adequações e a partir de descritores cuidadosamente selecionados, utilizamos diferentes bases de dados para compilar 154 artigos, 12 livros, bem como 5 websites, que atendiam aos critérios de inclusão previamente estabelecidos. Com isto, o potencial farmacológico da família Connaraceae ao nível mundial e suas possíveis contribuições ao desenvolvimento de novas identidades químicas, para uso humano, pode ser revisado e resumido numa única obra. Até aonde sabemos o artigo: "Connaraceae: Uma visão geral atualizada da pesquisa e do potencial farmacológico de 39 espécies" título em inglês "*Connaraceae: An updated overview of research and the pharmacological potential of 39 species"* é na atualidade a única publicação que sistematiza as informações acerca de Connaraceae já publicada.

Produzir um artigo de revisão, sistematizando um número considerável de diferentes táxons vegetais foi um processo desafiador, embora, fascinante e inspirador para as próximas etapas desse trabalho. Após reunir todas publicações foi possível evidenciar que muitas delas tratavam espécies sinônimas como plantas taxonomicamente distintas. Assim, a busca pela aceitabilidade do binômio científico atribuído para cada táxon (THE PLANT LIST, 2020), foi decisiva para que todas informações reunidas para Connaraceae pudessem ser resumidas num universo de 39 espécies. Dentre essas trinta e nove espécies, quatro taxons, *Agelaea pentagyna* (Lam.) Baill., *C. ferruginea, C. suberosus* e *R. minor,* encontram-se num estágio de desenvolvimento de pesquisa mais avançado que as demais, portanto, foram consideradas com o mais alto potencial farmacológico para atualidade. Assim *A. pentagyna, C. ferruginea, C. suberosus* e *R. minor* estão potencialmente mais próximas de dar origem a medicamentos. Por fim, através do artigo de revisão ampliamos nosso conhecimento a respeito das potencialidades farmacológicas para essa família de plantas. Em adição deixamos aos demais pesquisadores da área uma fonte de pesquisa acessível para Connaraceae, seus gêneros e espécies.

Foi através da construção do supracitado artigo, que evidenciamos que a utilização etnobotânica de Connaraceae em nível mundial compreende vários tipos de extração, dentre eles, a decocção, a infusão e a maceração (tintura/alcoolatura). Outros autores já conduziram trabalhos avaliando as diferenças no perfil de compostos com base em diferentes processos

extrativos (KONGKIATPAIBOON *et al.*, 2018; OLIVEIRA *et al.*, 2016; VEBER *et al.*, 2015). Com isto, surgiu a necessidade de se avaliar em Connaraceae se os diferentes processos de extração poderiam produzir algum efeito sobre o perfil quantitativo e/ou qualitativo de metabólitos secundários, compondo esse um segundo objetivo a ser alcançado.

Deste modo, o segundo o objetivo deste trabalho deu origem ao artigo: Quatro espécies quase inexploradas de *Connarus* brasileiro (Connaraceae): Composição química por ESI-QTof-MS / MS – GNPS e um potencial farmacológico, título em inglês "*Four almost unexplored species of Brazilian Connarus (Connaraceae): Chemical composition by ESI-QTof-MS/MS–GNPS and a pharmacologic potential"* A partir das espécies do gênero *Connarus*: os táxons *C. blanchetii, C. nodosus, C. regnellii,* e *C. suberosus* foram submetidos aos principais processos extrativos relatados em estudos com abordagem etnobotânica, e a partir destes conduzimos análises utilizando CL/MS a fim de avaliar o potencial farmacológico e a composição química dessas espécies. A composição química foi determinada a partir da construção de redes moleculares (YANG *et al*., 2013) como estratégia de desreplicação dos compostos químicos. A construção das redes moleculares foi conduzida a partir dos dados de CL/MS. Assim, através da simples visualização dos nodos produzidos nos diferentes clusters nestas redes moleculares foi possível avaliar se determinado composto podia ser detectado em cada um dos métodos. Até aonde sabemos essa é a primeira vez que redes moleculares são utilizadas como ferramenta para analisar a composição qualitativa de metabólitos secundários associados a diferentes processos extrativos. Adicionalmente, através deste trabalho, propomos a utilização de predição espectral (ALLEN *et al.*, 2014; DJOUMBOU-FEUNANG *et al.*, 2019) como um modelo complementar de análise de dados feita por redes moleculares. Essa iniciativa se mostrou muito útil na tentativa de identificação dos metabólitos secundários. Utilizando predição espectral foi possível, a partir dos espectros de massas dos compostos anotados, determinar quais íons fragmento estão relacionados a um modelo predito de fragmentação teórica para determinado composto de interesse. Assim, a predição espectral permitiu adicionar um maior grau de certeza a respeito das identidades químicas propostas pelas redes moleculares, tendo sido determinante para a identificação de 23 compostos a partir das 4 espécies. Ainda com base nos dados desse artigo e frente aos processos extrativos foi possível determinar que a maioria dos metabólitos, do ponto de vista qualitativo, pode ser identificada igualmente entre os 3 métodos. Entre os principais metabólitos secundários identificados os flavonoides camferol, quercetina e miricetina e seus heterosídeos deram origem aos principais clusters associados a essas espécies vegetais. A avaliação do potencial farmacológico foi conduzida a partir de uma breve revisão

de literatura para alguns compostos incluindo (quercetina, miricetina, guaijaverina, hiperina, miricetrina e querciturona). As principais atividades relacionadas a estes compostos incluem efeitos antioxidantes e anti-inflamatórios, bem como, efeitos inibitórios sobre enzimas relacionadas ao metabolismo de carboidratos e atividades antiglicantes. Portanto, a partir da publicação desse artigo o número de espécies de Connaraceae cuja composição química foi avaliada em nível mundial subiu de 10 para 13 espécies. Três novos e inexplorados táxons *C. blanchetii, C. nodosus* e *C. regnellii* antes negligenciados foram apresentados a comunidade científica e o potencial farmacológico associado à composição química pode ser apresentado.

A terceira etapa deste trabalho foi realizar fracionamento com diferentes solventes orgânicos a partir dos macerados etanólico obtidos das folhas de *C. blanchetii*, *C. regnellii, C. suberosus* e *R. glazioui*. A partir das diferentes frações obtidas, determinamos quantitativamente o teor de polifenois, flavonoides e taninos e conduzimos um screening de atividade antioxidante com DPPH$^{\bullet}$. Aferimos que as frações acetato de etila e n-butanólica mostraram-se as mais ricas nessas classes de metabólitos químicos e a partir dessas buscamos avaliar a atividade antioxidante através de ensaios com DPPH$^{\bullet}$ e FRAP. Os resultados combinados da avaliação quantitativa de metabólitos com a atividade antioxidante mostram que as frações n-butanólicas de *C. blanchetii*, *C. regnellii* e *C. suberosus* em adição as frações acetato de etila e n-butanólica de *R. glazioui* apresentavam um perfil favorável quanto ao potencial em inibir a glicação proteica. Portanto, a partir dessas frações avaliamos a inibição da glicação de proteínas pelas vias oxidativa e não oxidativa e determinamos a composição química a utilizando CL/EM onde os espectros produzidos foram analisados através de ferramentas de bioinformática. Ao final dessa fase identificamos 29 compostos entre as espécies *C. blanchetii*, *C. regnellii, C. suberosus* e *R. glazioui*. Na avaliação da atividade antiglicante pela via oxidativa *C. suberosus* e *R. glazioui* frações n-butanólica e acetato de etila respectivamente mostraram-se as mais ativas. Para a via não oxidativa *R. glazioui* - acetato de etila e *C. blanchetii* – n-butanol apresentaram o melhor desempenho. Dentre os metabólitos identificados para essas espécies de Connaraceae, vários flavonoides e outros polifenólicos estão implicados como moléculas promissoras úteis no controle de complicações do diabetes mediadas pela glicação de proteínas e pelo desequilíbrio do metabolismo redox. Portanto, considerando que no diabetes, a hiperglicemia resulta em um aumento na produção de radicais livres por um mecanismo que envolve a oxidação da glicose seguida da glicação de proteínas e que os processos mitocondriais estão associados a exacerbação do estresse oxidativo produzindo ao final muitas das complicações desta doença compostos de origem que reduzam

um o mais destes eventos podem ser extremamente úteis. Destaca-se que a busca de novas alternativas terapêuticas, com atividade antioxidate e antiglicante pode reduzir as complicações diabéticas e, portanto, trazer inúmeros benefícios no tratamento complementar aos hipoglicemínas orais e a insulina. Finalmente, considerando essas quatro espécies de Connaraceae identificamos que *R. glazioui* e *C. suberosus* têm o mais amplo rol de metabólitos potencialmente úteis no tratamento de complicações associadas ao diabetes, incluindo os compostos apigenina, camferol, quercetina, miricetina, ácido clorogênico, hiperina e rutina dentre outros, embora o potencial das outras espécies não possa ser descartado.

A quarta e última etapa desse trabalho foi avaliar o efeito inibitório dos extratos de Connaraceae sobre duas enzimas relacionadas ao metabolismo de carboidratos. Nesta fase, a partir das frações n-butanólicas de todas as espécies em adição a fração acetato de etila de *R. glazioui* estudamos a ação inibitória frente a α-amilase e α-glicosidase. Para α-amilase, os extratos provenientes de *R. induta* - folhas e *C. suberosus* - *folhas* e cascas apresentaram os melhores desempenhos, por outro lado, na avaliação com α-glicosidase *C. regnellii* - *folhas* teve o melhor resultado. Frente aos resultados obtidos α-amilase e α-glicosidase, para as condições experimentais ensaiadas, nenhum alcançou mais do que 50% de inibição e, portanto, optamos por não darmos continuidade a essa avaliação. Na sequência estabelecemos a composição química dos extratos utilizando os métodos já referendados nas outras fases desse trabalho e encontramos a partir dessas espécies um extenso rol de flavonoides e outros compostos químicos com alto potencial farmacológico associado. Entre as frações n-butanólicas obtidas das folhas de *C. blanchetii*, *C. nodosus, C. regnellii, C. suberosus, R. glazioui* e *R. induta* 35 metabólitos tiveram duas identidades reveladas. Para as cascas, nesta mesma fração, em *C. regnellii*, *C. suberosus* e *R. cuspidata* outros quatro foram identificados. Por fim, na avaliação da fração acetato de etila em *R. glazioui*, 10 compostos tiveram sua identidade proposta.

De posse de todas as informações produzidas nas fases experimentais deste trabalho e dos dados provenientes da revisão da literatura optamos por estudar o perfil toxicológico dos extratos de *C. suberosus*, *R. glazioui* e *R. cuspidata.* Utilizando uma linhagem de células endoteliais avaliamos os percentuais de viabilidade celular frente as concentrações de 10, 25, 50 e 100 µg/mL de extrato. Nesta avaliação, nenhuma espécie reduziu os percentuais de viabilidade celular em comparação aos controles, fato que denota que para essas condições experimentais, não há indícios de toxicidade celular. Portanto, infere-se, de modo preliminar, que os extratos associados a essas espécies não apresentam toxicidade, embora, experimentos adicionais e complementares ainda precisam ser conduzidos. Na sequência, uma possível

reversão da citotoxicidade mediada pelos extratos, quando células endoteliais expostas são expostas a altas concentrações de glicose foi avaliada. Nesta etapa foi observado que para a maioria das espécies as concentrações de 10 e 25 µg/mL são capazes de reverter o efeito citotóxico. Assim é possível inferir que a reversão da citotoxicidade está provavelmente associada ao efeito sinérgico dos diferentes compostos fenólicos principalmente dos flavonoides presentes nessas espécies de plantas. Entre os possíveis mecanismos pelo quais esses efeitos são observados os efeitos antioxidantes dos metabólitos secundários não pode ser descartado.

6 CONCLUSÕES

- Estudando Connaraceae ratificamos o enorme potencial farmacológico associado a biodiversidade brasileira, pois conforme foi possível demonstrar, essa é uma família de plantas seriamente impactada pela ação antrópica, principalmente frente as áreas de *hotspots* de Biodiversidade, e ainda pouco estudada pela comunidade científica.
- Em nível mundial algumas espécies de Connaraceae estão em num nível mais avançado de pesquisas científicas tais como *A. pentagyna*, *C. ferruginea*, *C. suberosus* e *R. minor* e portanto, encontram-se mais próximas de dar origem a novos medicamentos.
- Utilizando redes moleculares e outras ferramentas de bioinformática foi possível determinar a composição química associada aos principais métodos extrativos tradicionalmente utilizados (decocção, infusão e maceração) e verificar que do ponto de vista qualitativo a maioria dos compostos encontra-se presente frente aos três métodos.
- Através do fracionamento com diferentes solventes orgânicos encontramos que as frações mais polares dos extratos de Connaraceae apresentam o melhor perfil quantitativo de compostos e estão relacionadas com o melhor potencial antioxidante.
- As frações n-butanólicas das folhas de *C. blanchetii* e *C. suberosus* em adição a fração acetato de etila de *R. glazioui* apresentam alta capacidade de inibir a glicação proteica pela via oxidativa.
- As frações n-butanólicas das folhas de *C. blanchetii*, *C. regnellii*, *C. suberosus* e n-butanólica/acetato de etila de *R. glazioui* apresentam alta capacidade de inibir a glicação proteica pela via não- oxidativa.
- Na avaliação da toxicidade frente a células endoteliais em meio normoglicêmico *C. suberosus* e *R. cuspidata* – frações n-butanólicas e *R. glazioui* – fração acetato de etila e n-butanol não se apresentam tóxicas para nenhuma das concentrações testadas.
- As concentrações mais baixas dos extratos 10 e 25 µg/mL de *Connarus suberosus*, *Rourea cuspidata* e *Rourea glazioui* mostram-se capazes de reverter a citotoxicidade induzida pela hiperglicemia em células endoteliais.
- Combinando diferentes plataformas de bioinformática foi possível identificar a presença de mais de 30 diferentes metabólitos secundários entre as sete espécies de Connaraceae estudadas.
- Quatro espécies de Connaraceae *C. blanchetii*, *C. nodosus*, *C. regnellii* e *R. glazioui* contidas em áreas de Hotspots de biodiversidade e antes negligenciadas foram apresentadas a comunidade científica e seu potencial farmacológico pode ser aferido.

- Finalmente, através desse trabalho evidenciamos o enorme potencial farmacológico de Connaraceae e suas espécies como potenciais fontes de moléculas úteis no tratamento do diabetes e suas complicações e destacamos que esses e outros táxons dessa família merecem novos e outras abordagens científicas.

7 PERSPECTIVAS

- O estudo das atividades farmacológicas para essas espécies de Connaraceae terá continuidade através da pesquisa de doutorado da Colega Aline. Serão abordadas questões quantitativas para os principais metabólitos associados às espécies, abordagens toxicológicas e farmacológicas no modelo i*n vivo*.
- Num futuro trabalho questões pretendemos ampliar o uso de CL/EM e ferramentas de bioinformática para estabelecer a composição química de mais espécies de Connaraceae e através desses dados auxiliar os taxonomistas na correta identificação dos gêneros e espécies.
- O estudo do metabolismo vegetal secundário através de ferramentas analíticas como CL/EM pode ser no futuro usado para auxiliar na taxonomia vegetal.
- Outras espécies de Connaraceae precisam ser estudadas quanto ao seu potencial farmacológico.

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APÊNDICES

- Informação de suporte artigo 1 a 3
- Certidões SISGEN

Supplementary Files

Connaraceae: An updated overview of research and the pharmacological potential of 39 species.

Luís Fernando Nunes Alves Paim, Cássio Augusto Patrocínio Toledo, Joicelene Regina Lima da Paz, Aline Picolotto, Guilherme Ballardin, Vinicius Castro Souza, Mirian Salvador, Sidnei Moura.

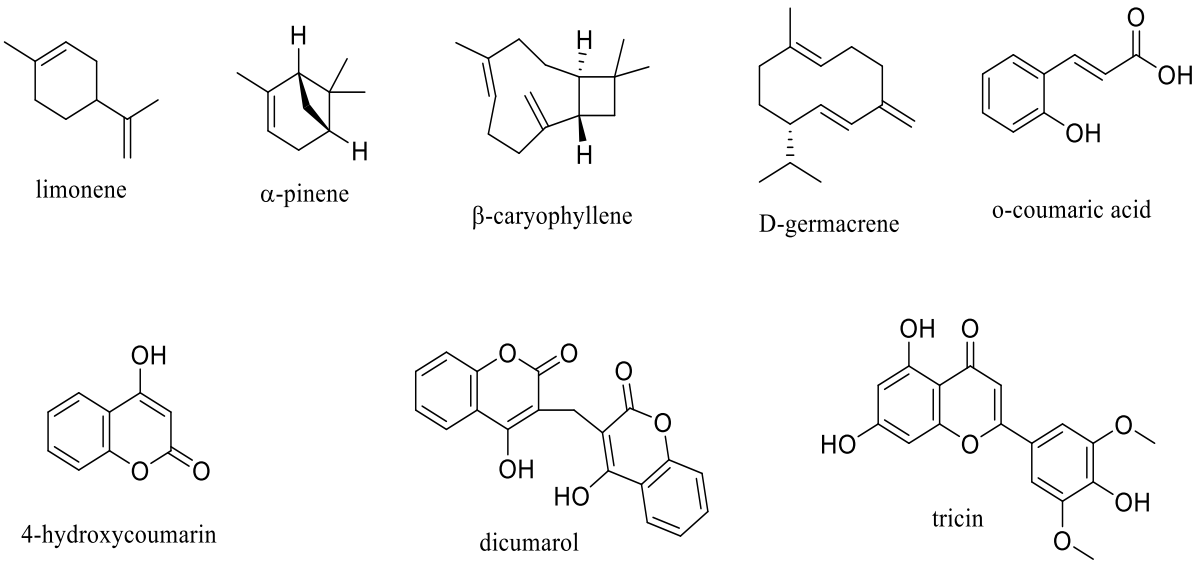

Figure 6: Structures of chemical constituents identified in the genus *Agelaea*.

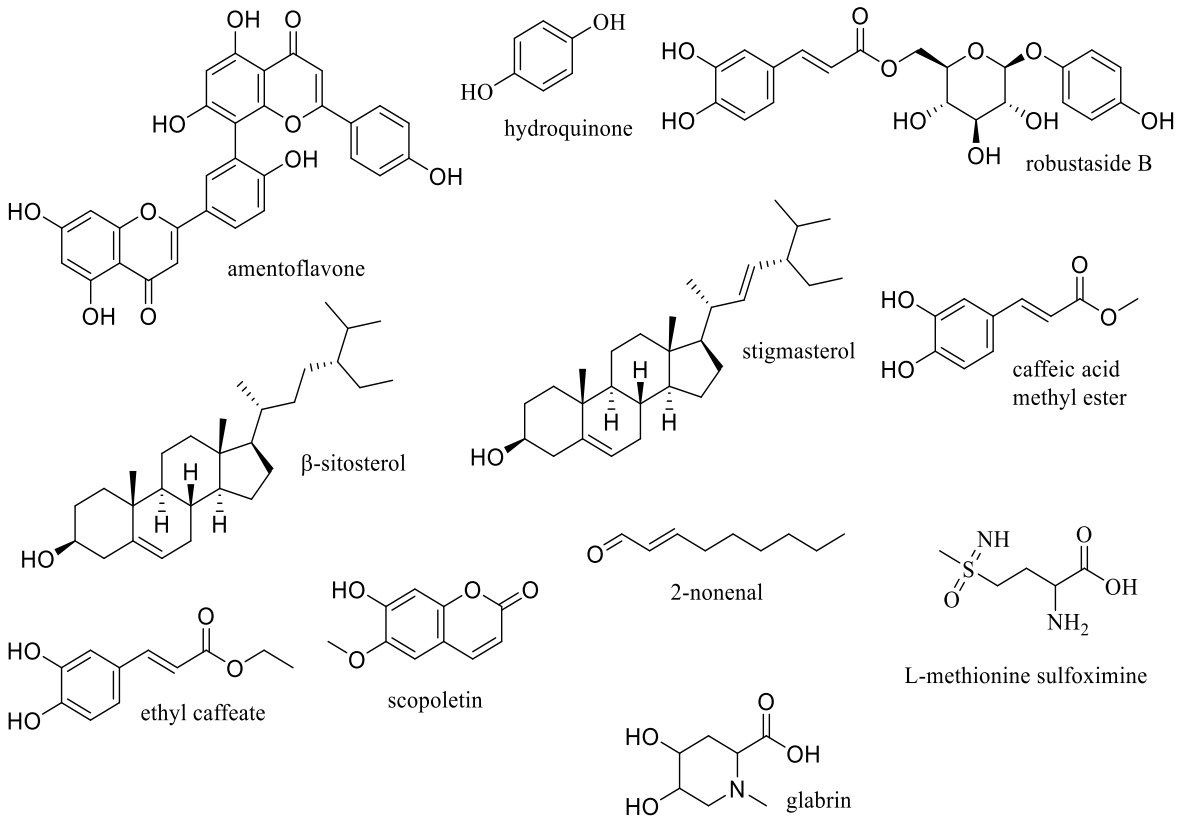

Figure 7: Structures of chemical constituents identified in the genus *Cnestis*.

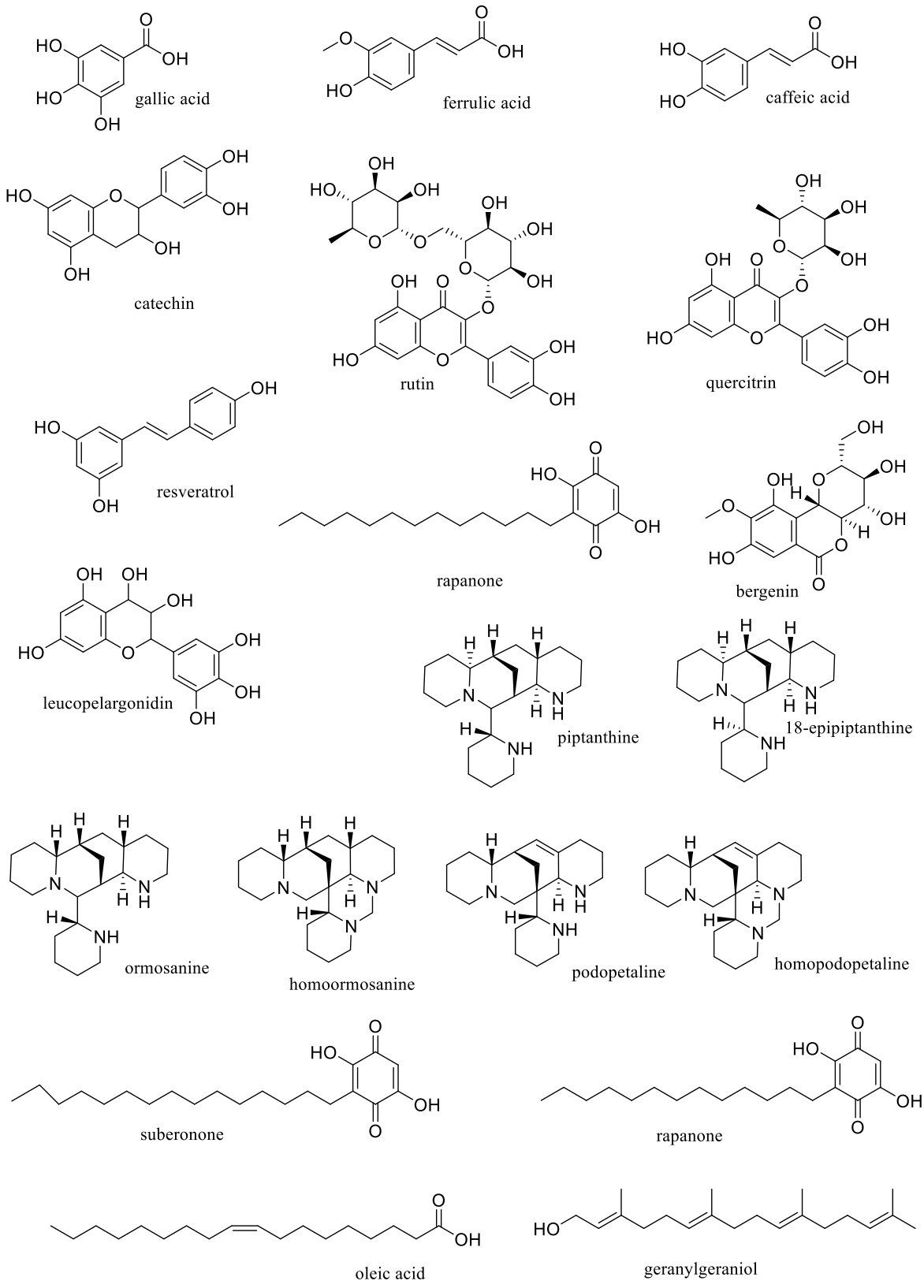

Figure 8: Structures of chemical constituents identified in the genus *Connarus*.

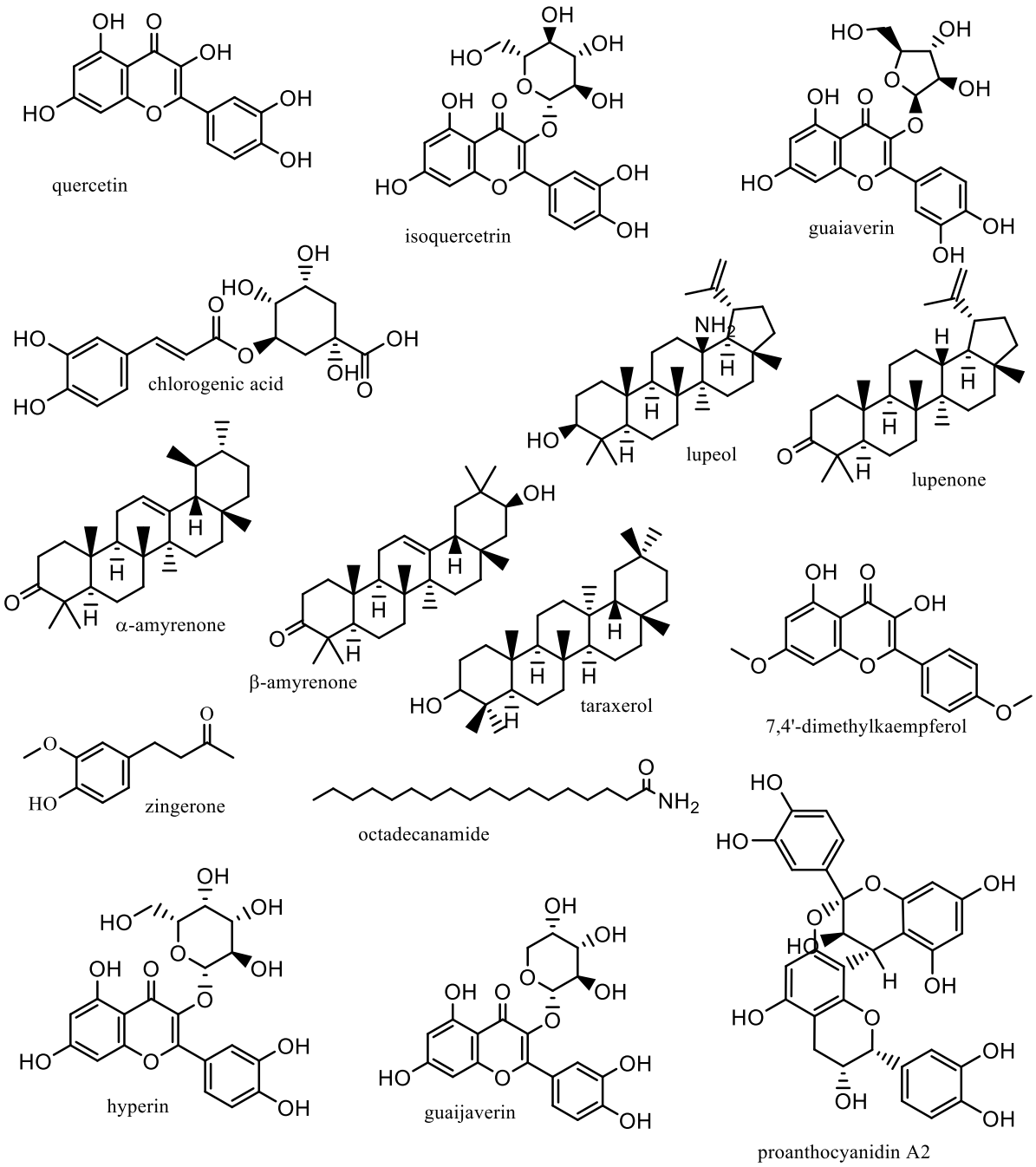

Figure 9a: Structures of chemical constituents identified in the genus *Rourea*.

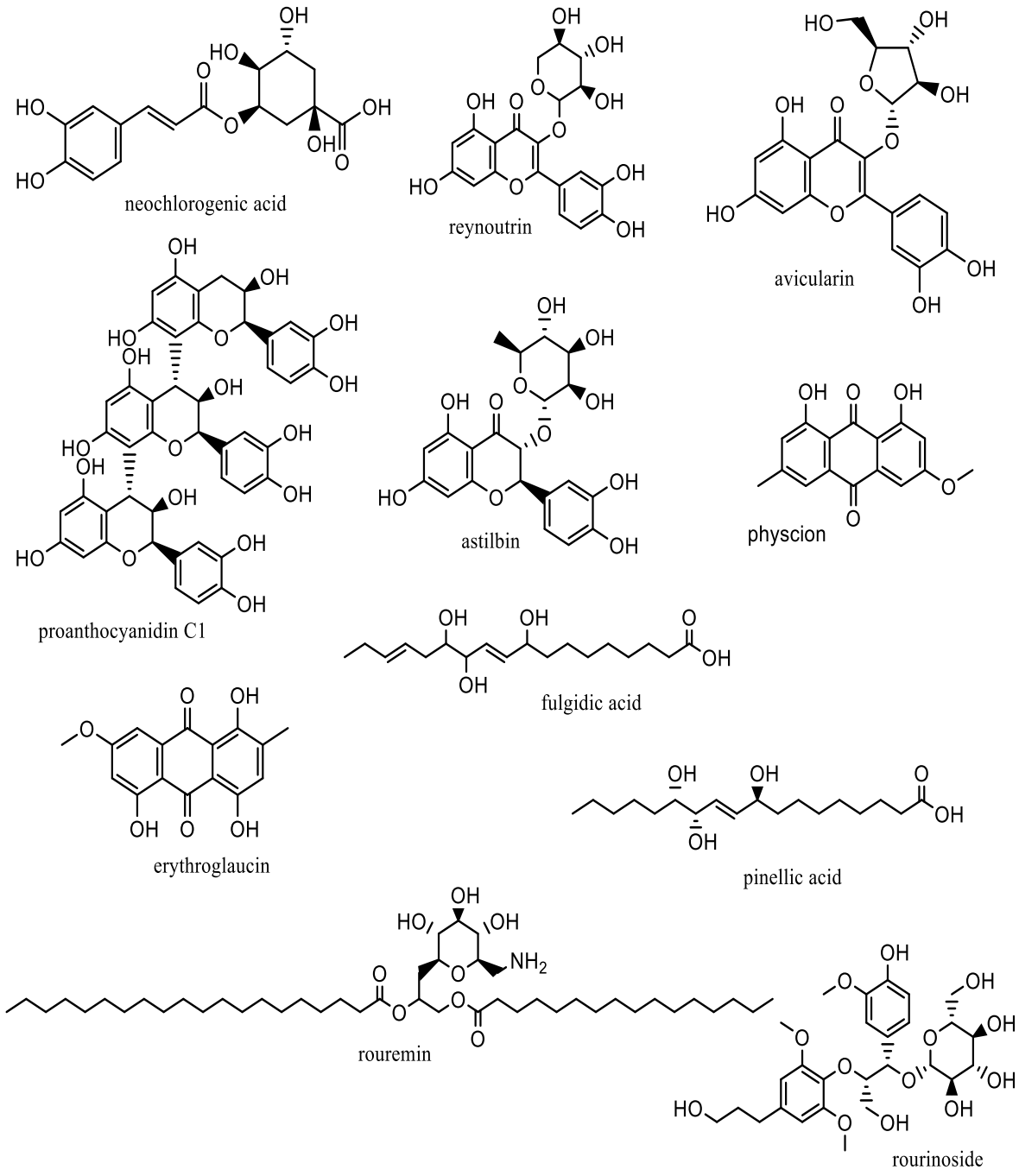

Figure 9b (continued): Structures of chemical constituents identified in the genus *Rourea*.

Four almost unexplored species of Brazilian *Connarus* (Connaraceae): chemical composition by ESI-QTof-MS/MS – GNPS and a pharmacologic potential

Luís Fernando Nunes Alves Paim[a], Paulo Roberto dos Santos[a], Cássio Augusto Patrocínio Toledo[b], Luana Minello[c], Joicelene Regina Lima da Paz[d], Vinicius Castro Souza[e], Mirian Salvador[c], Sidnei Moura[a]

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Standards

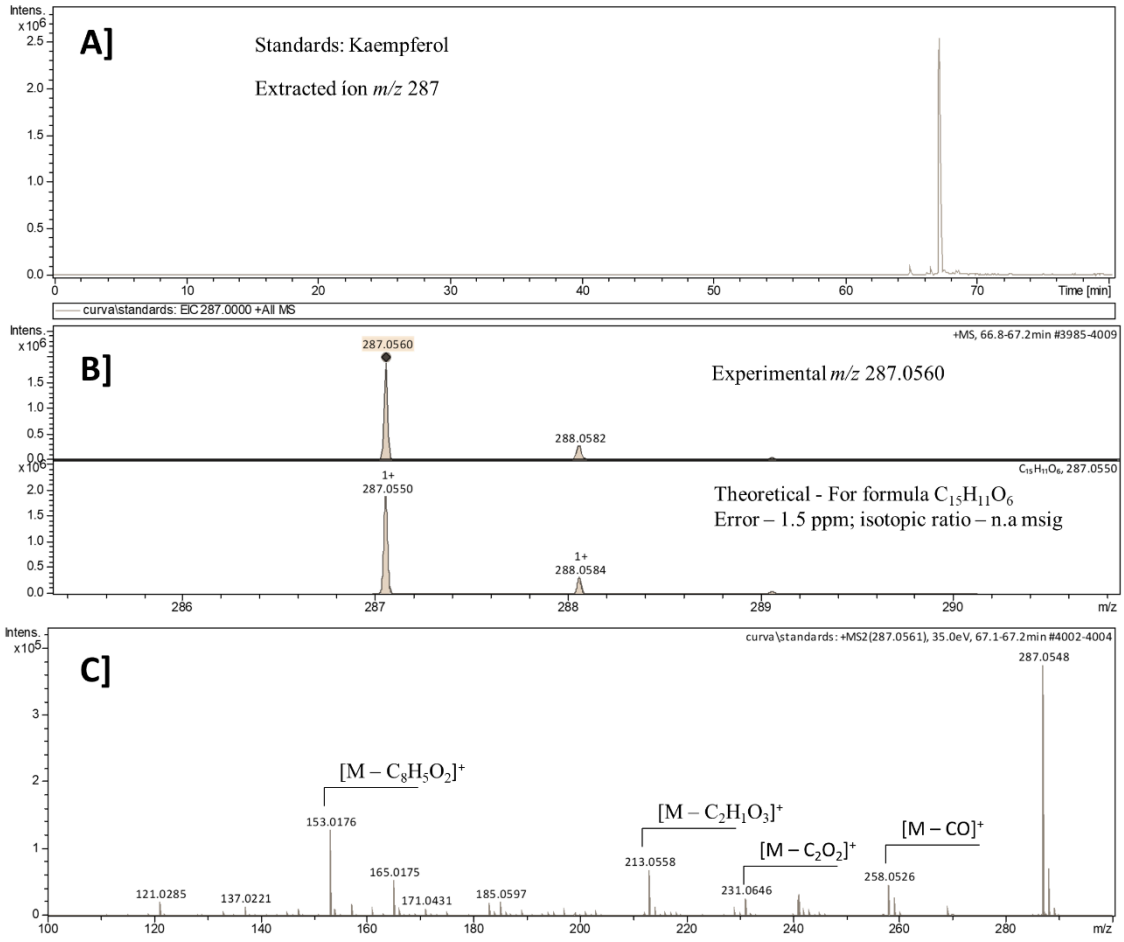


Figure 1: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 287.0560 in MS-MS mode HRESIMS spectrum.

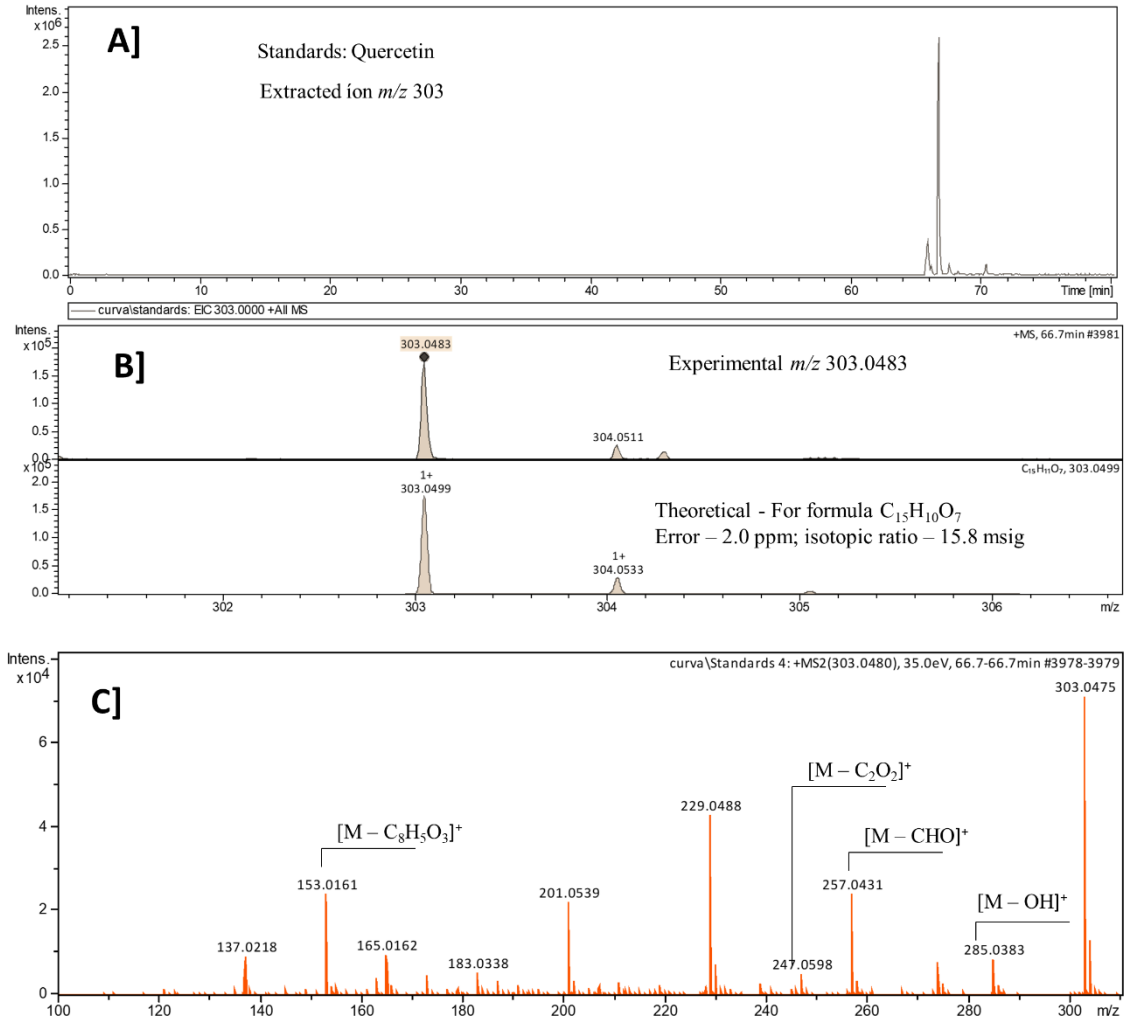


Figure 2: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 303.0483 in MS-MS mode HRESIMS spectrum.

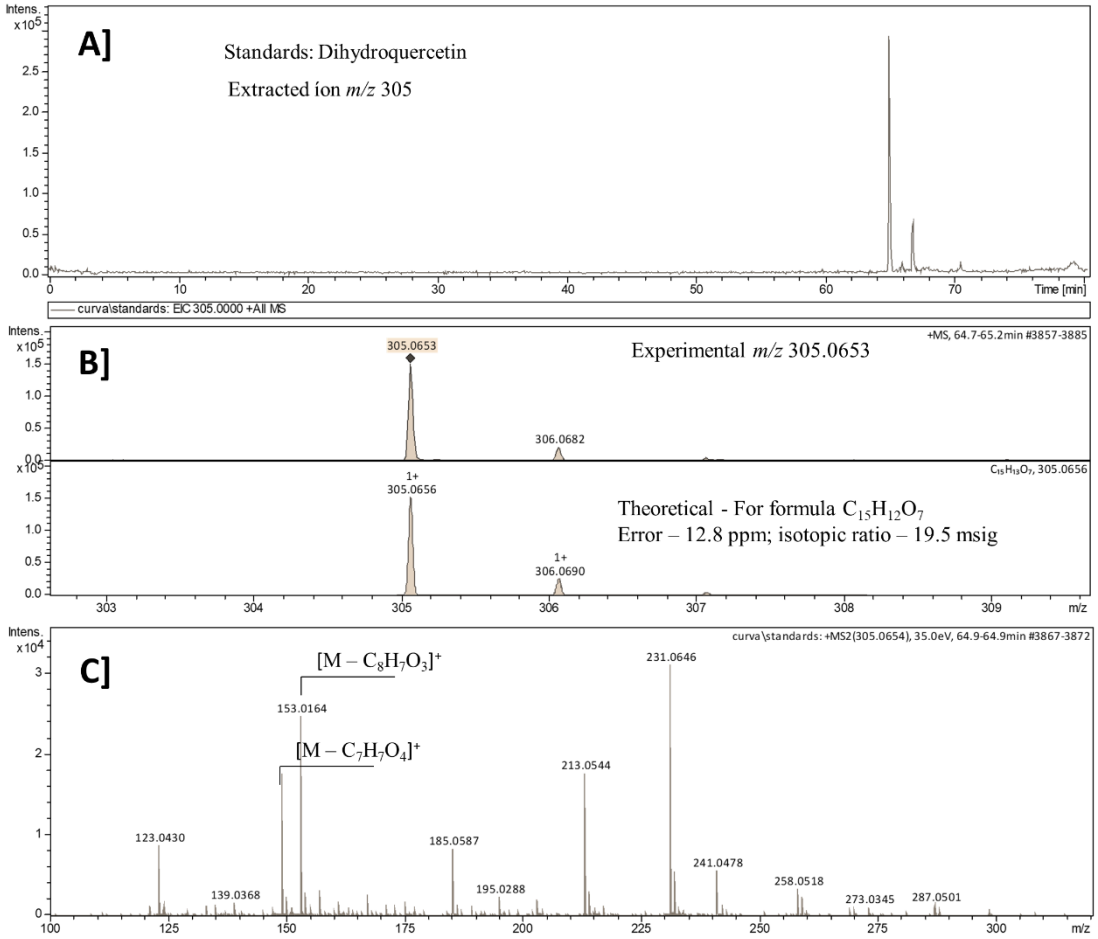


Figure 3: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 305.0653 in MS-MS mode HRESIMS spectrum.

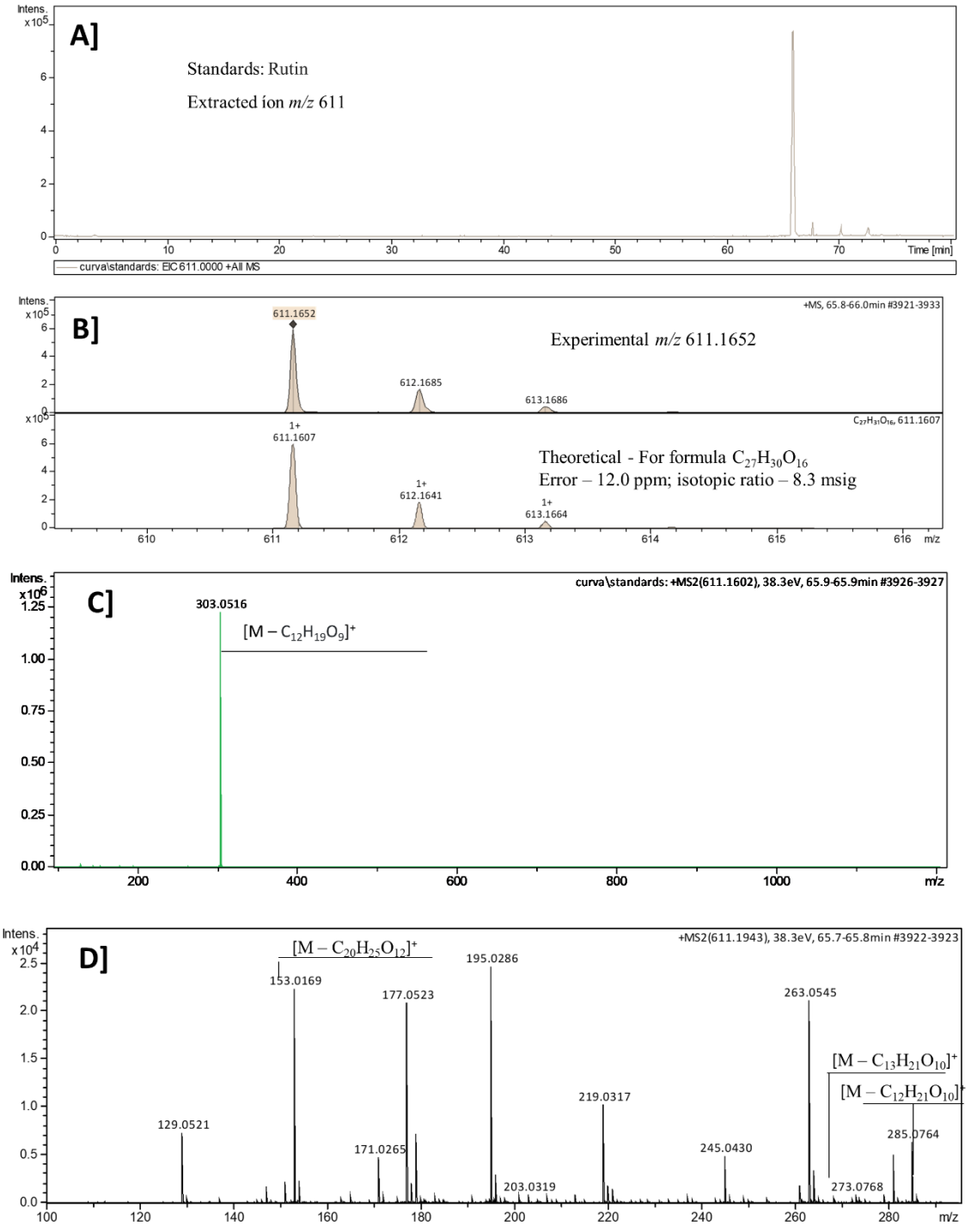


Figure 4: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 611.1652 in MS-MS mode HRESIMS spectrum.

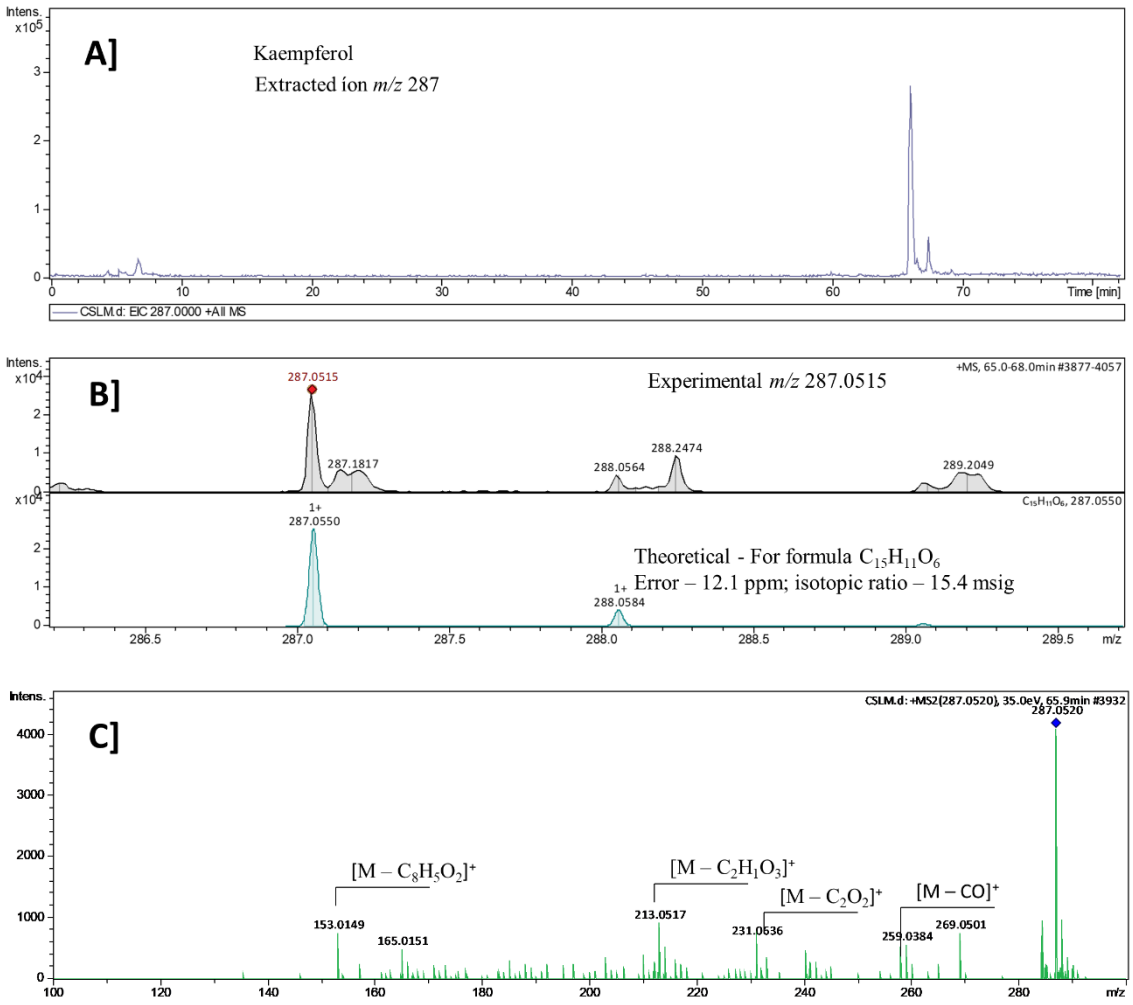


Figure 5: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 287.0511 in MS-MS mode HRESIMS spectrum.

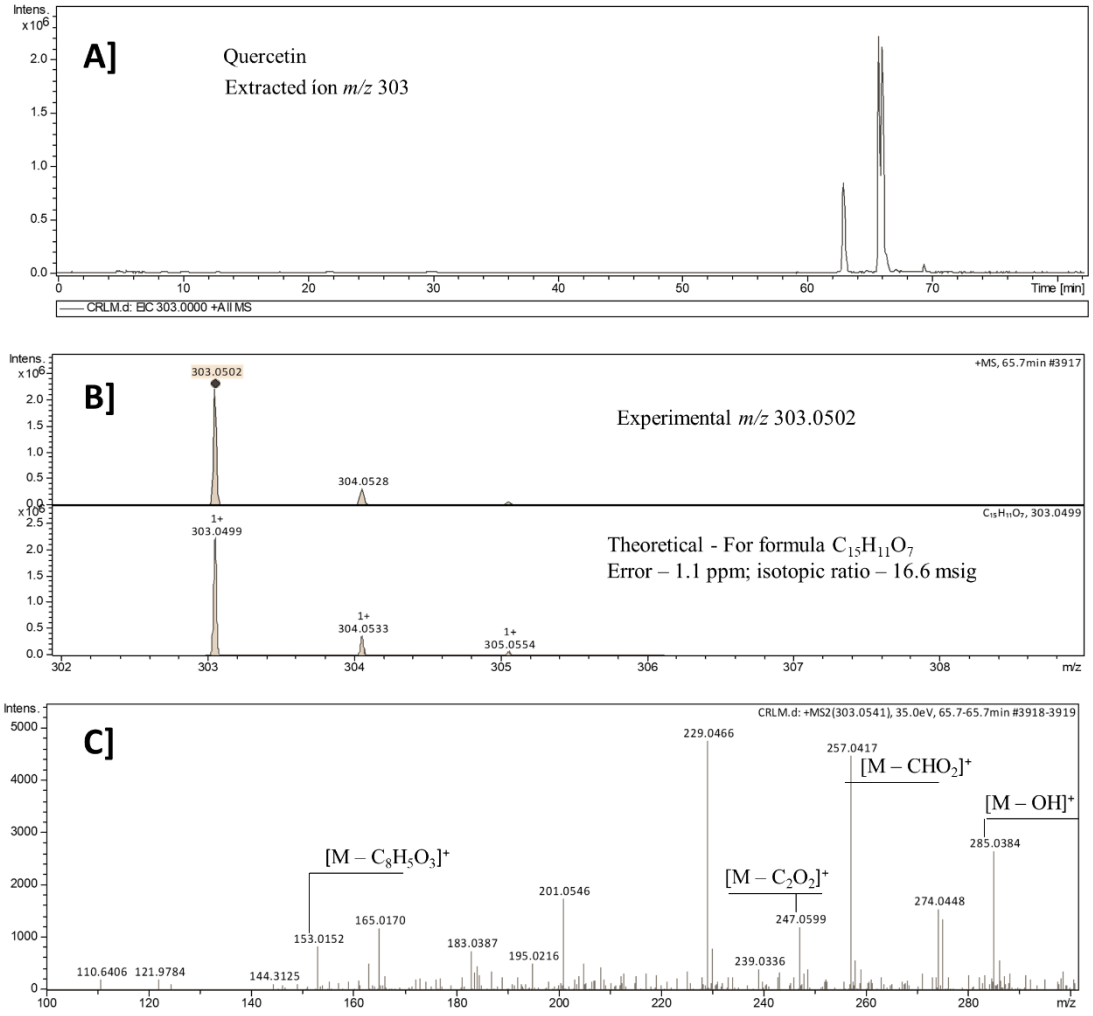


Figure 6: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 303.0502 in MS-MS mode HRESIMS spectrum.

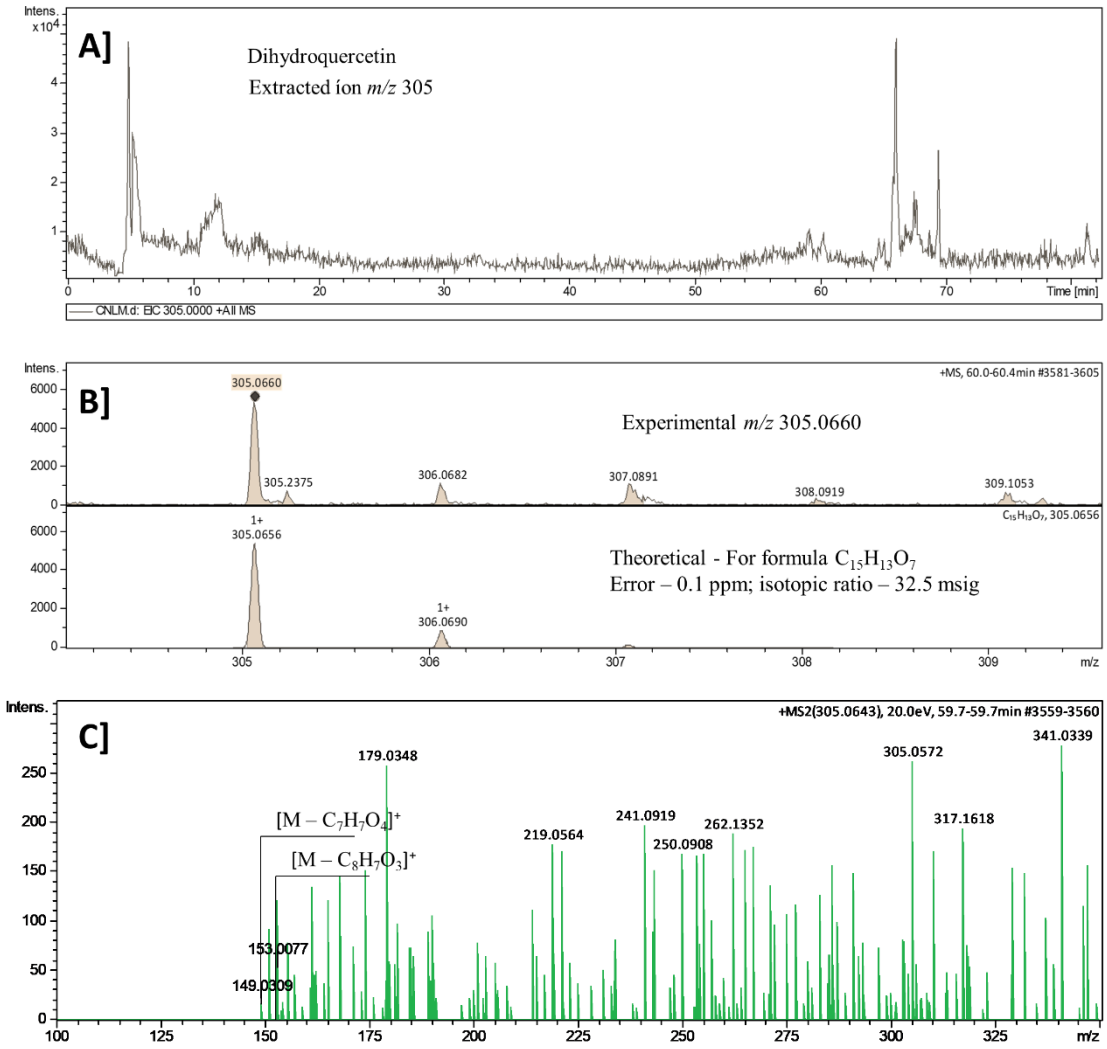


Figure 7: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 305.0600 in MS-MS mode HRESIMS spectrum.

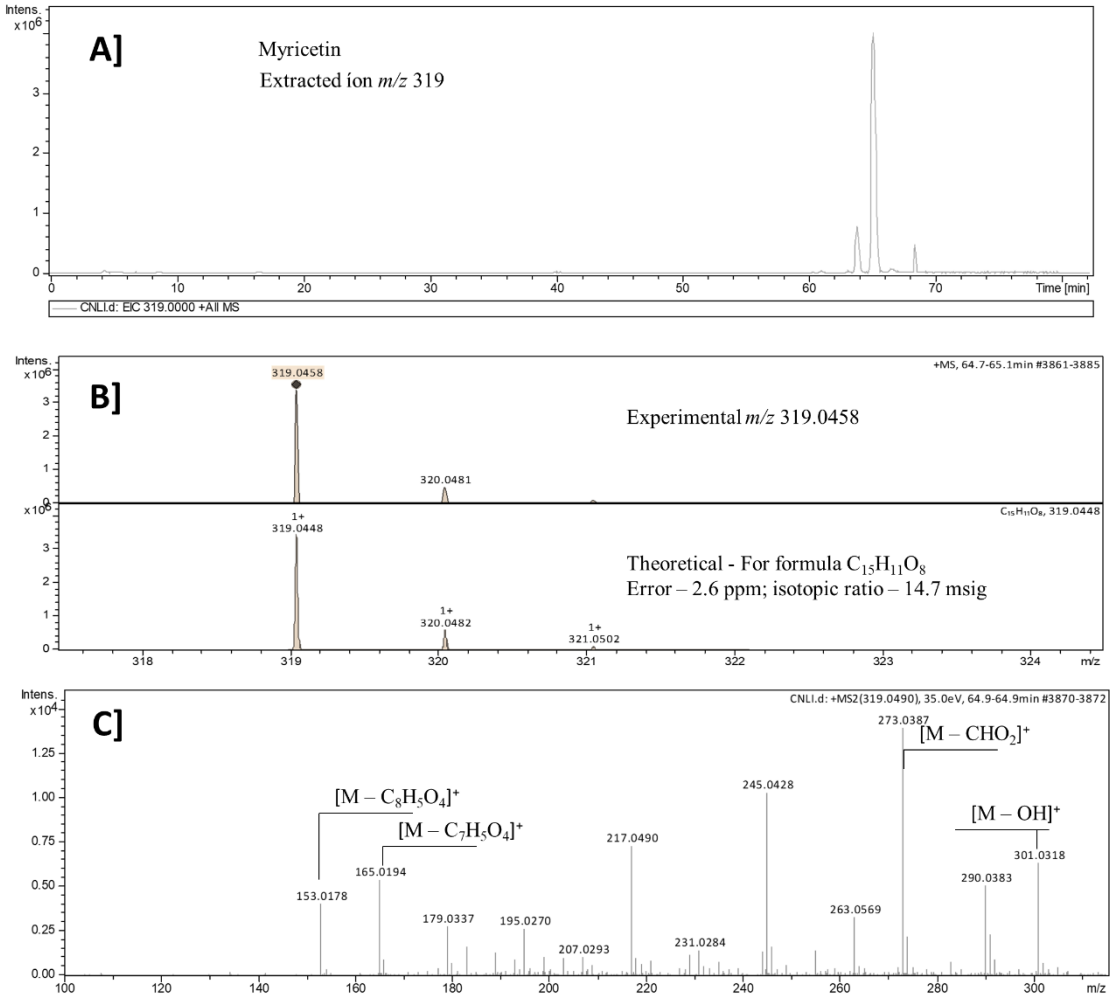


Figure 8: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 319.0458 in MS-MS mode HRESIMS spectrum.

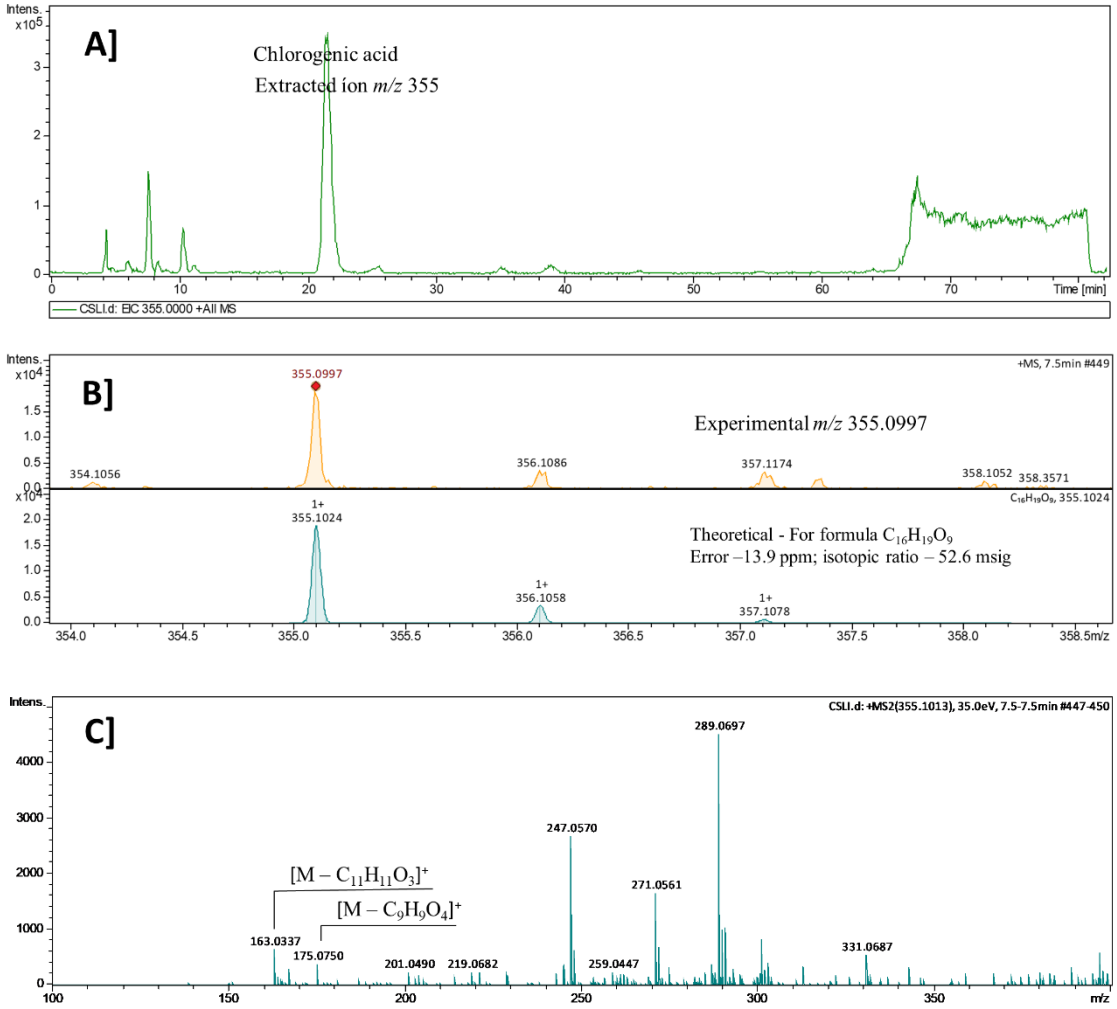


Figure 9: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 355.0997 in MS-MS mode HRESIMS spectrum.

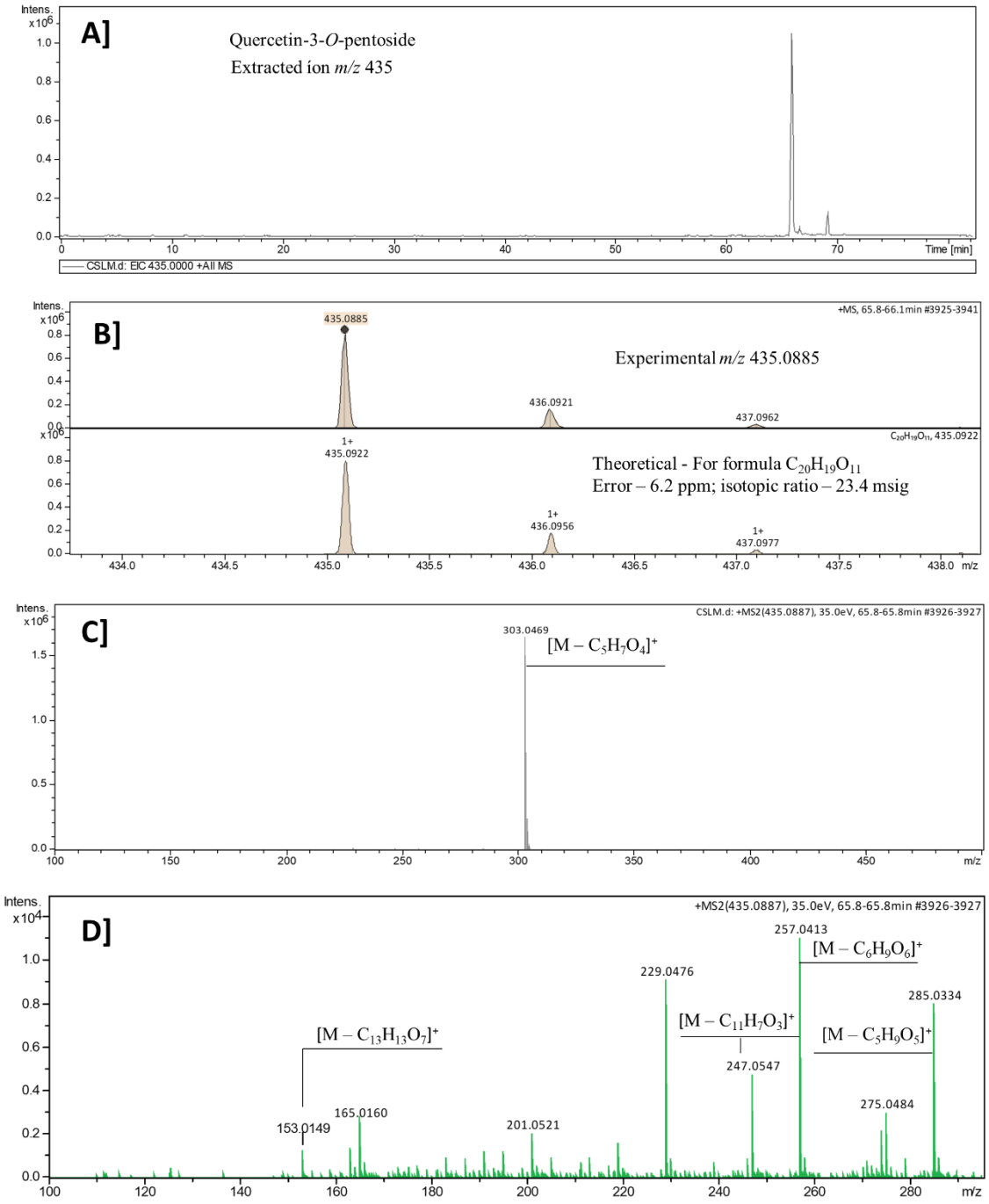


Figure 10: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 435.0885 in MS-MS mode HRESIMS spectrum.

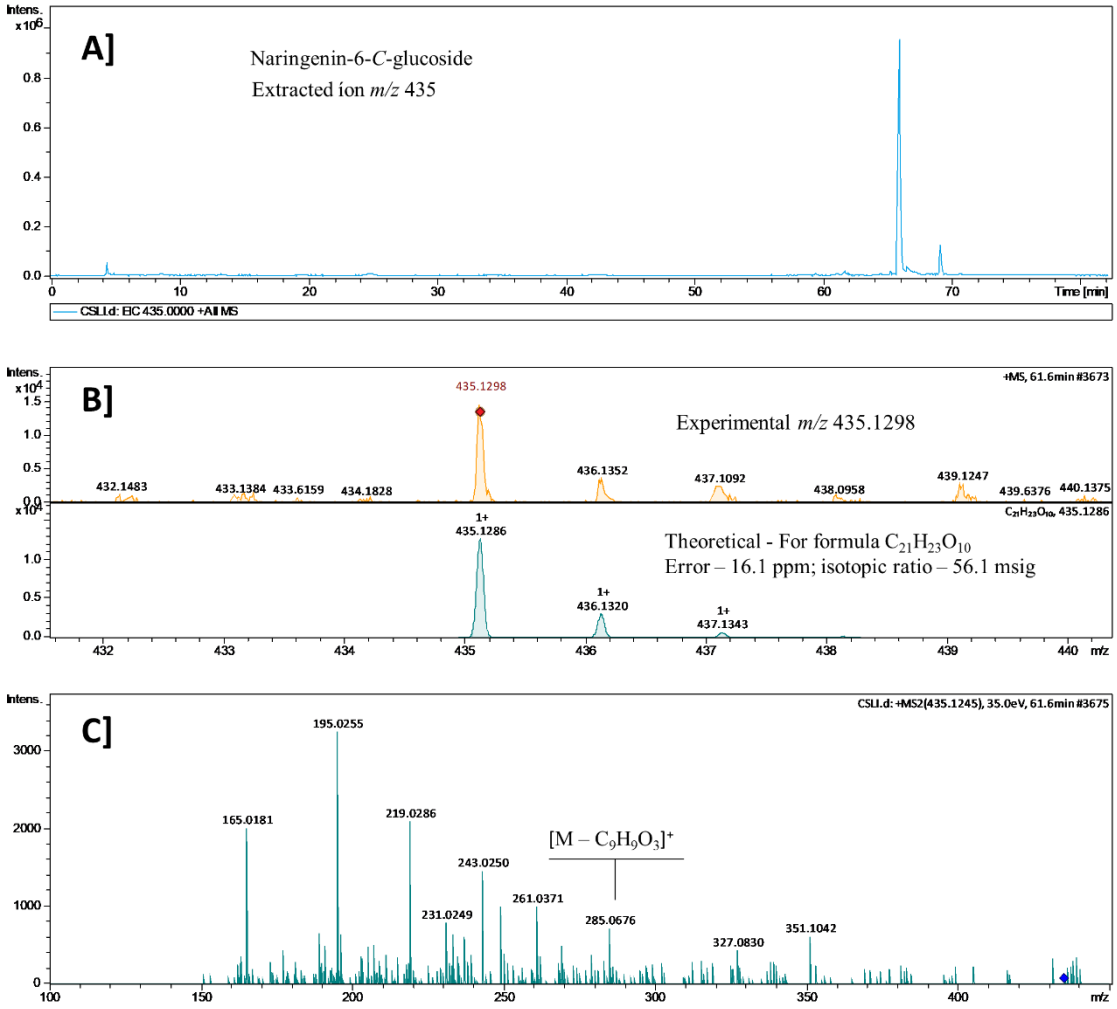


Figure 11: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 435.1298 in MS-MS mode HRESIMS spectrum.

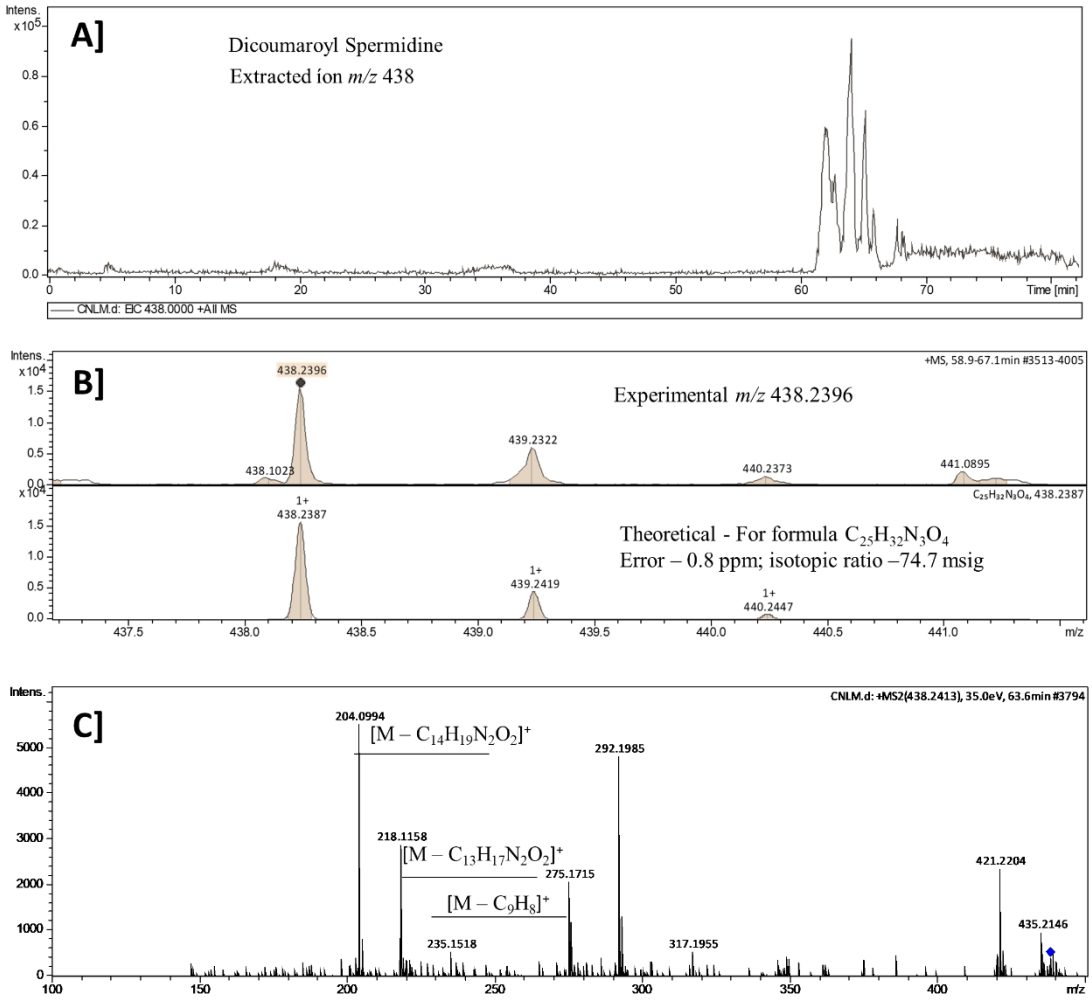


Figure 12: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 438.2396 in MS-MS mode HRESIMS spectrum.

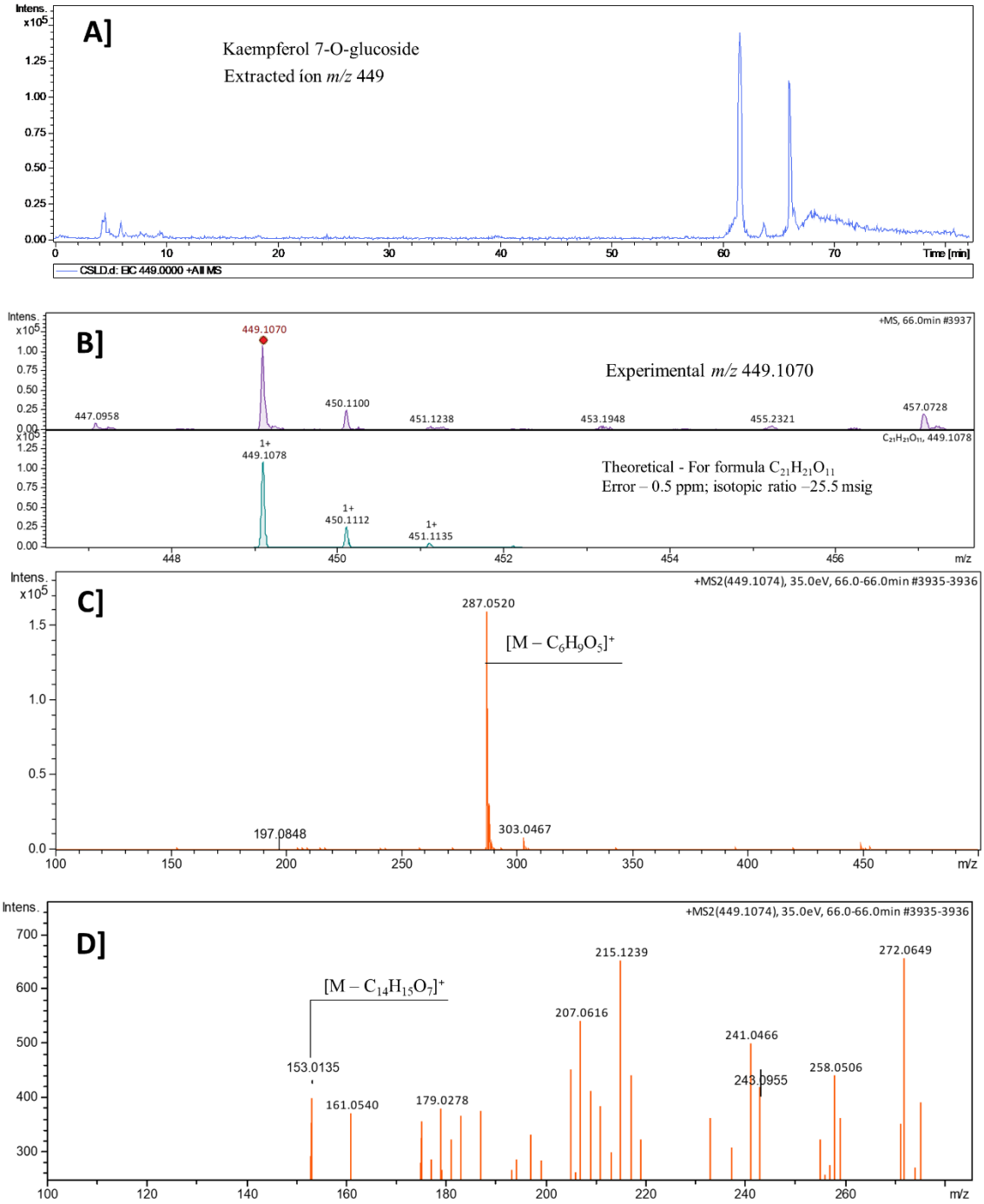


Figure 13: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 449.1070 in MS-MS mode HRESIMS spectrum.

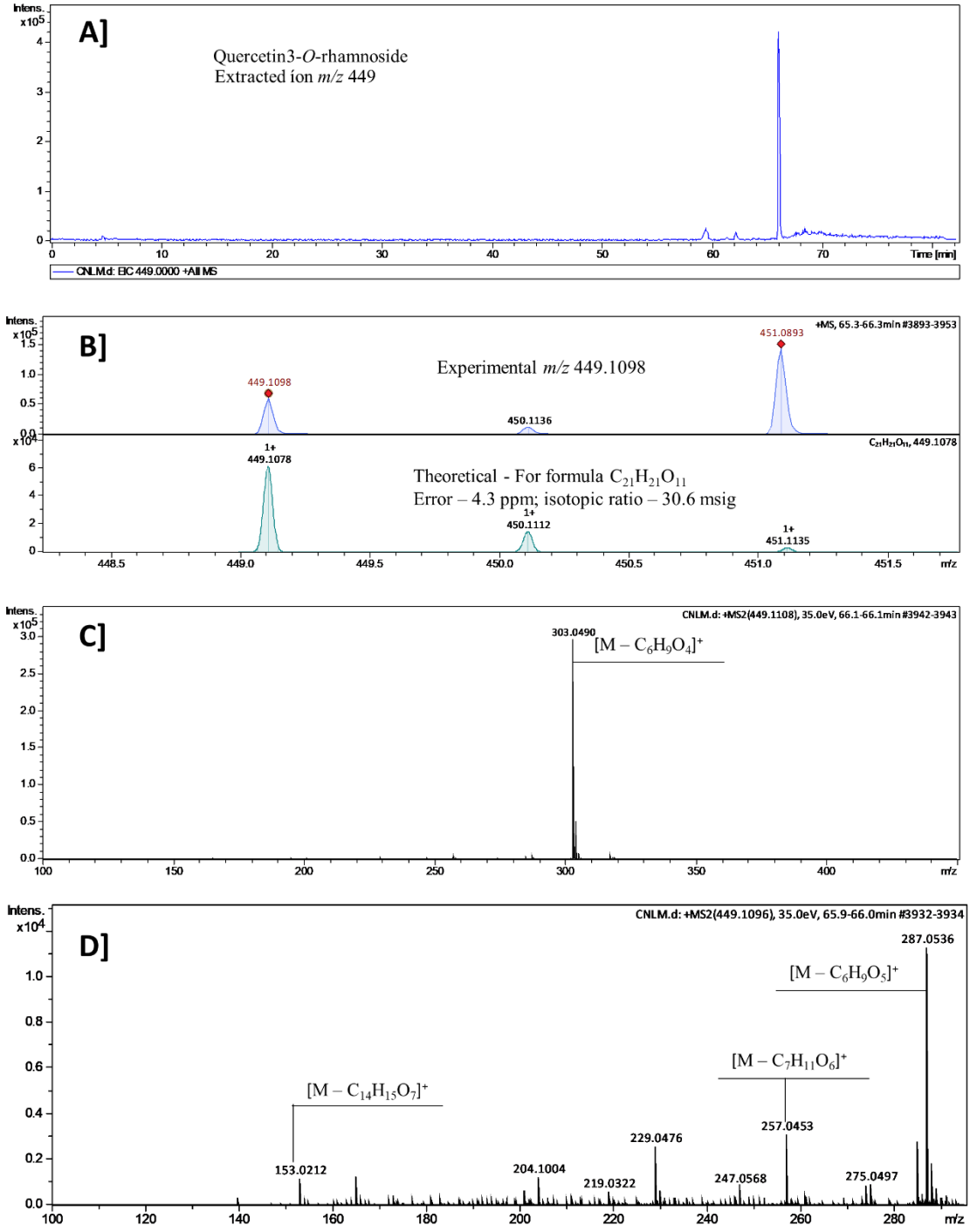


Figure 14: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 449.1098 in MS-MS mode HRESIMS spectrum.

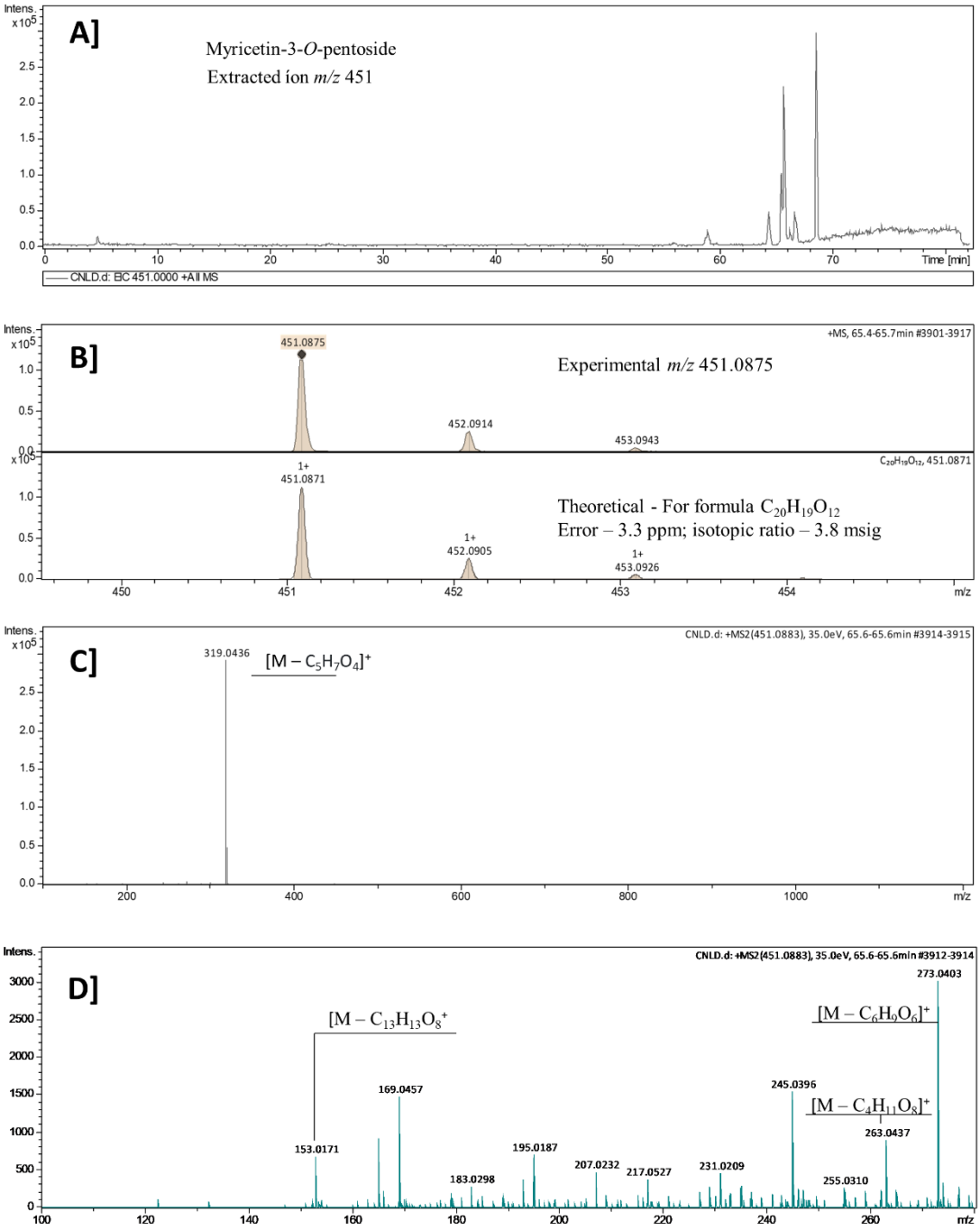


Figure 15: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 451.0875 in MS-MS mode HRESIMS spectrum.

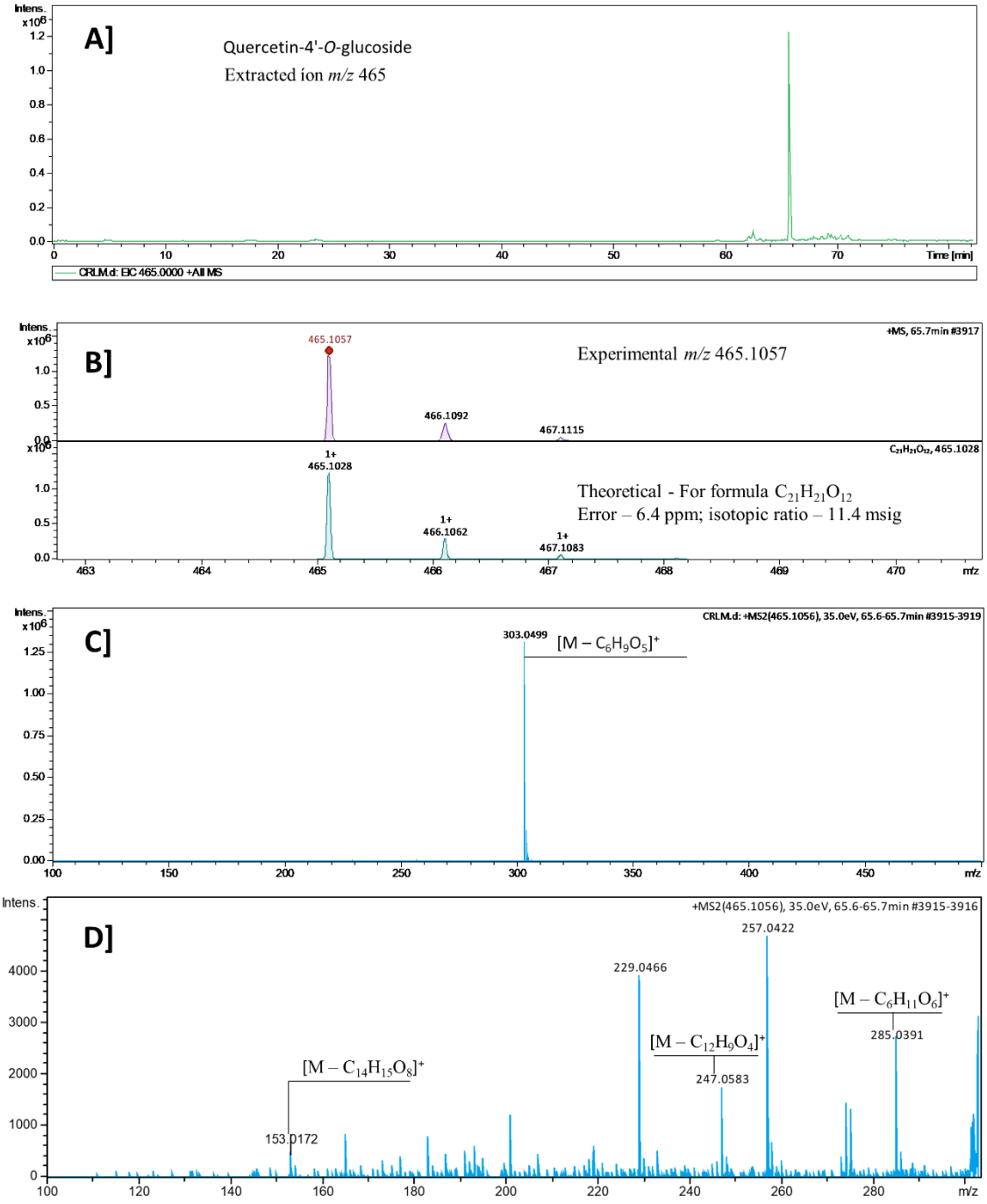


Figure 16: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 465.1057 in MS-MS mode HRESIMS spectrum.

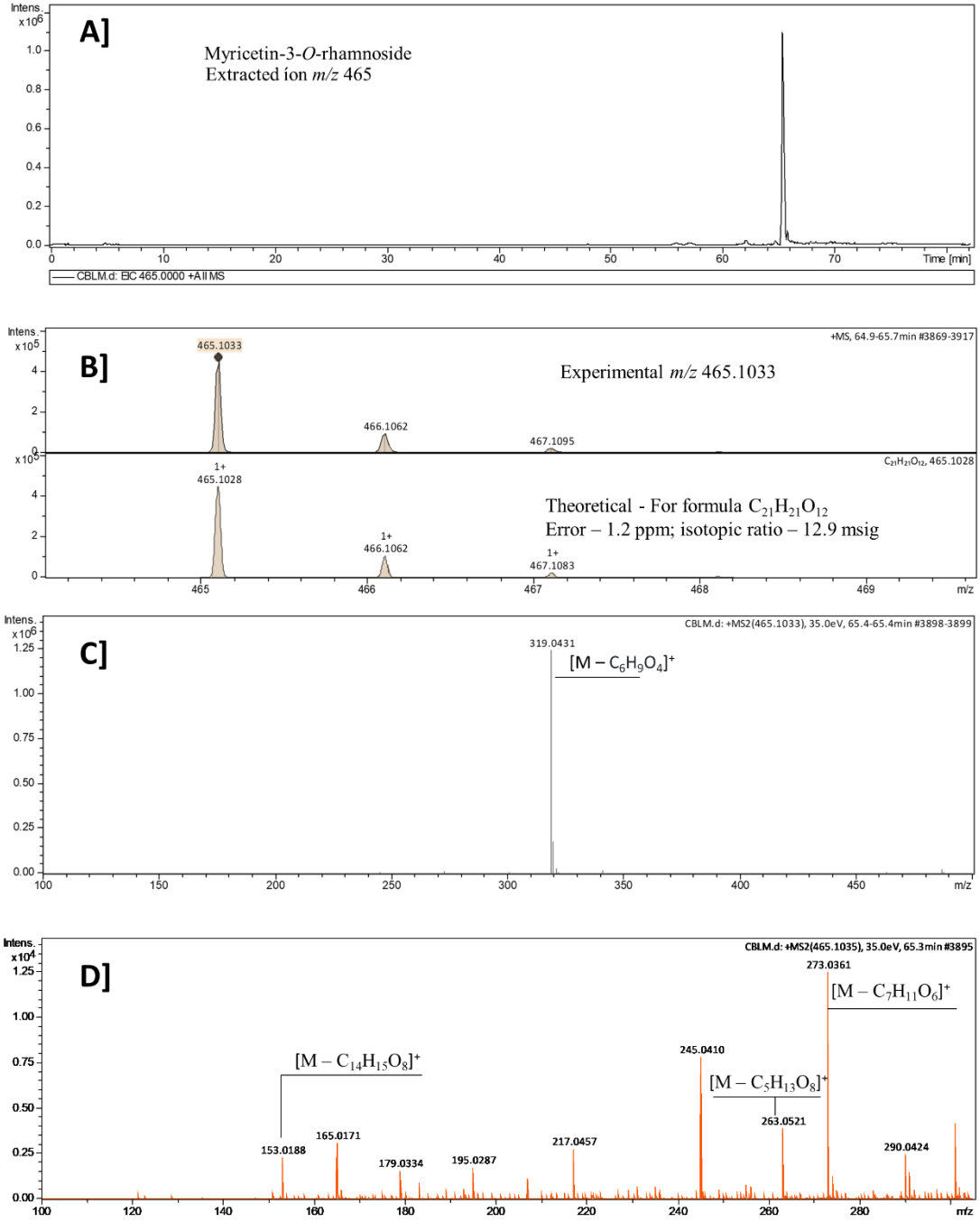


Figure 9: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 465.1033 in MS-MS mode HRESIMS spectrum.

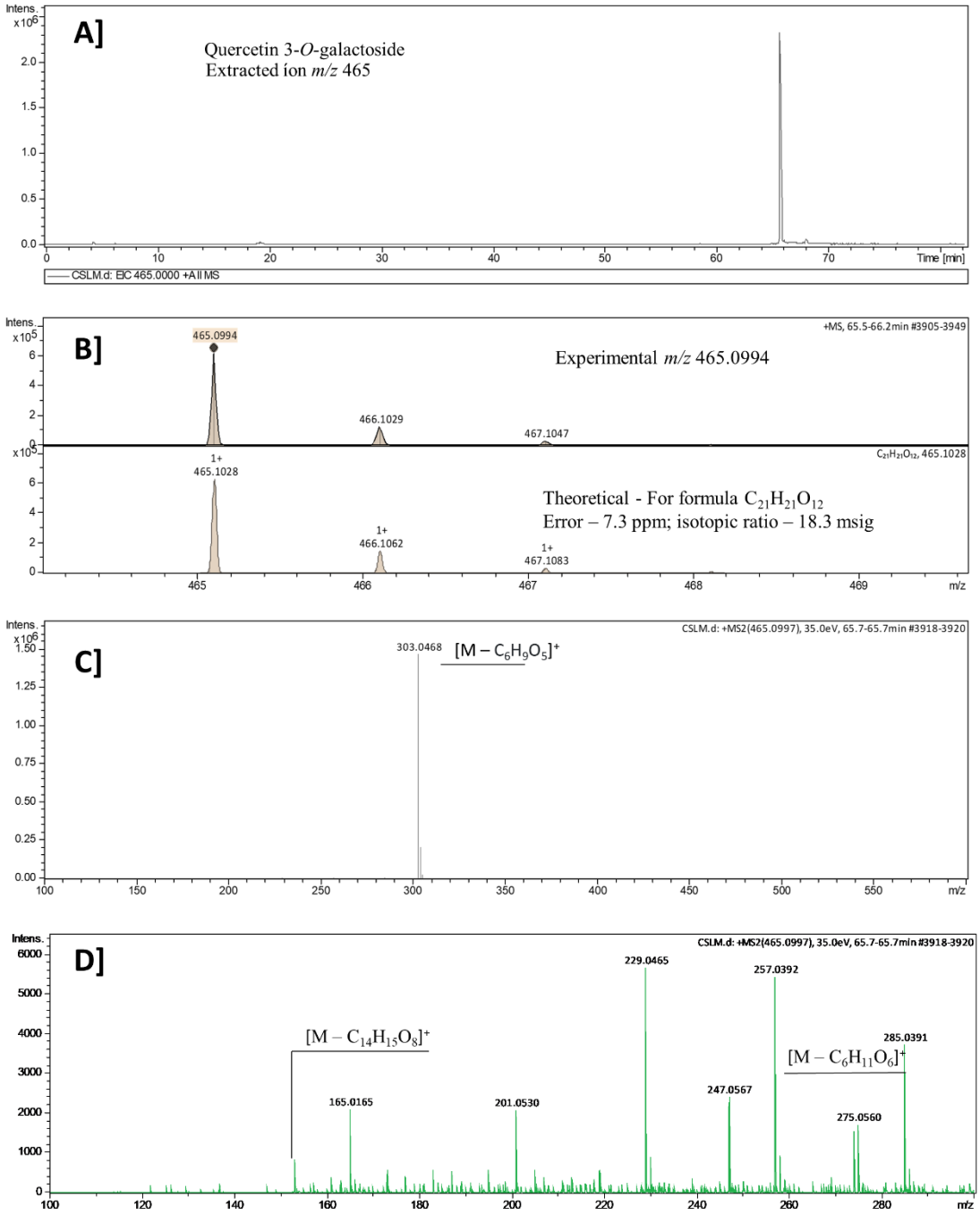


Figure 18: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 465.0994 in MS-MS mode HRESIMS spectrum.

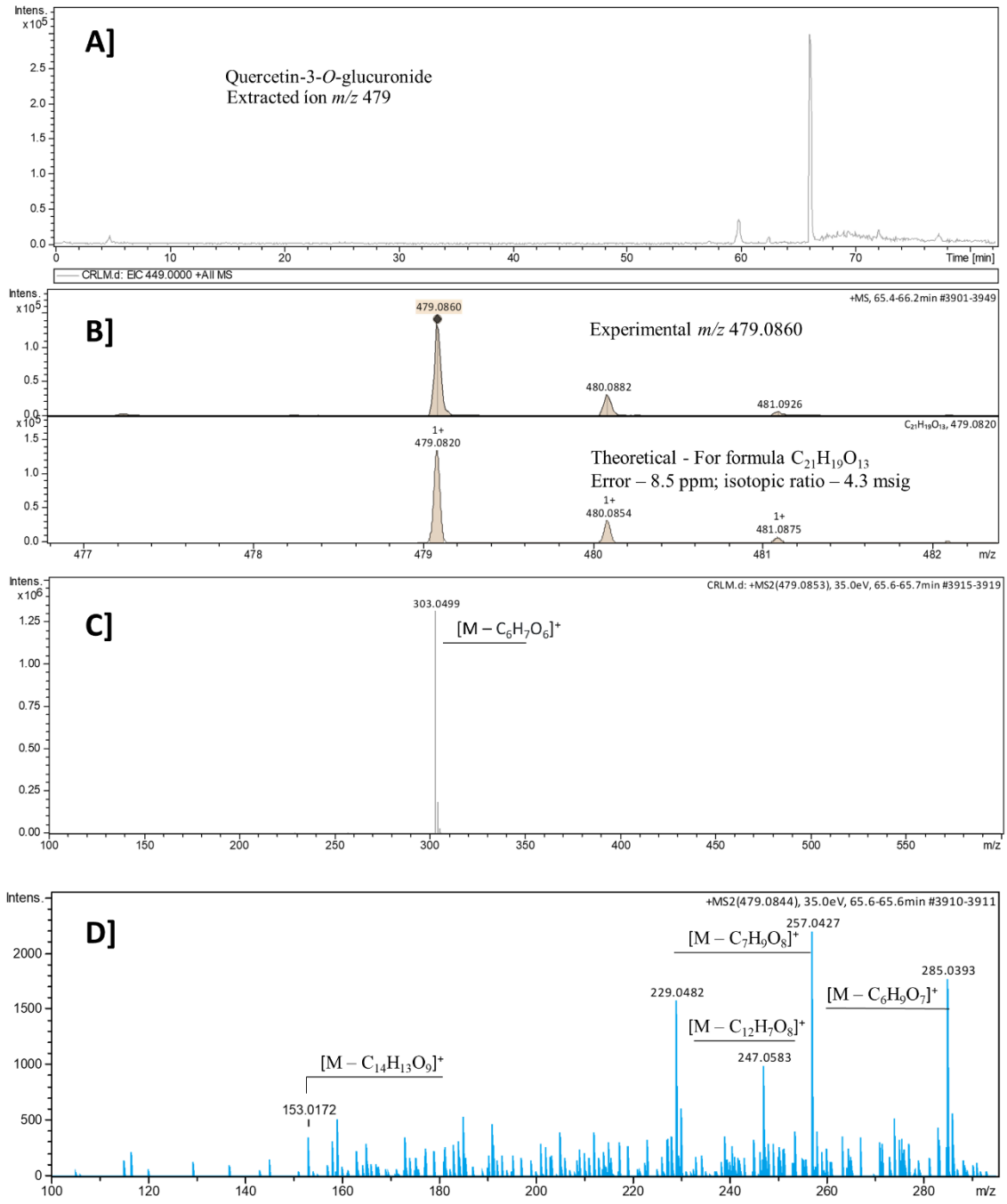


Figure 19: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 479.0860 in MS-MS mode HRESIMS spectrum.

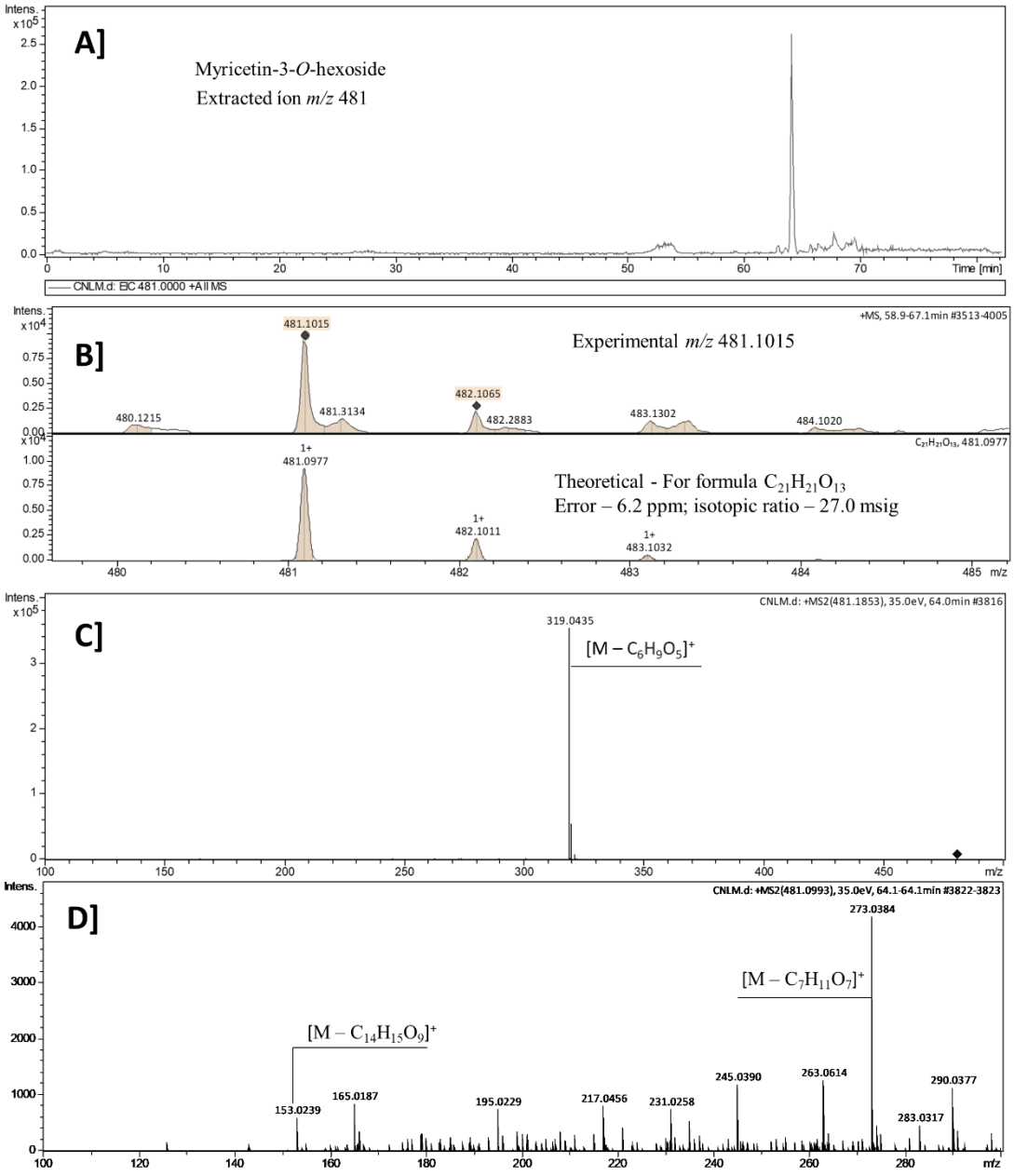
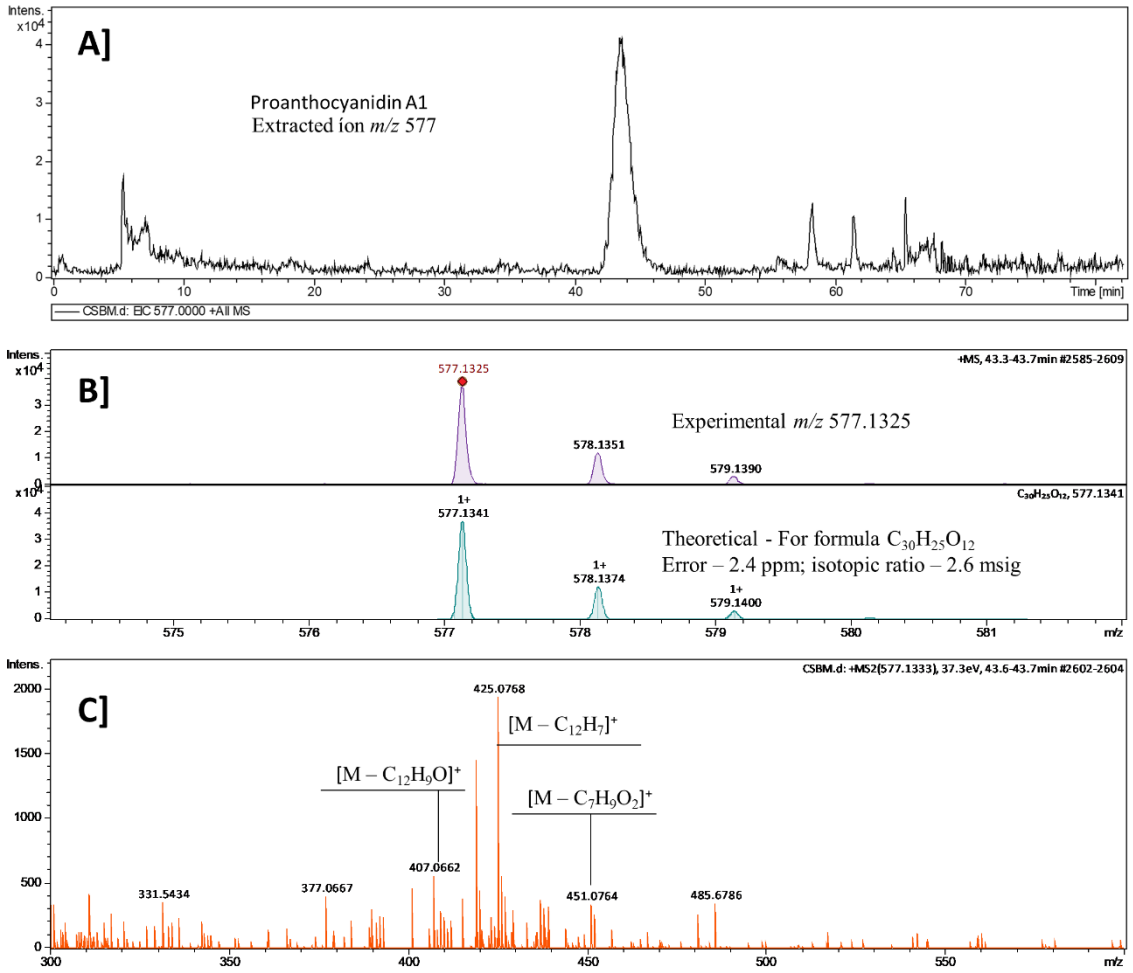


Figure 100: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 481.1015 in MS-MS mode HRESIMS spectrum.



127

128 Figure 111: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
129 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 577.1325 in MS-
130 MS mode HRESIMS spectrum.

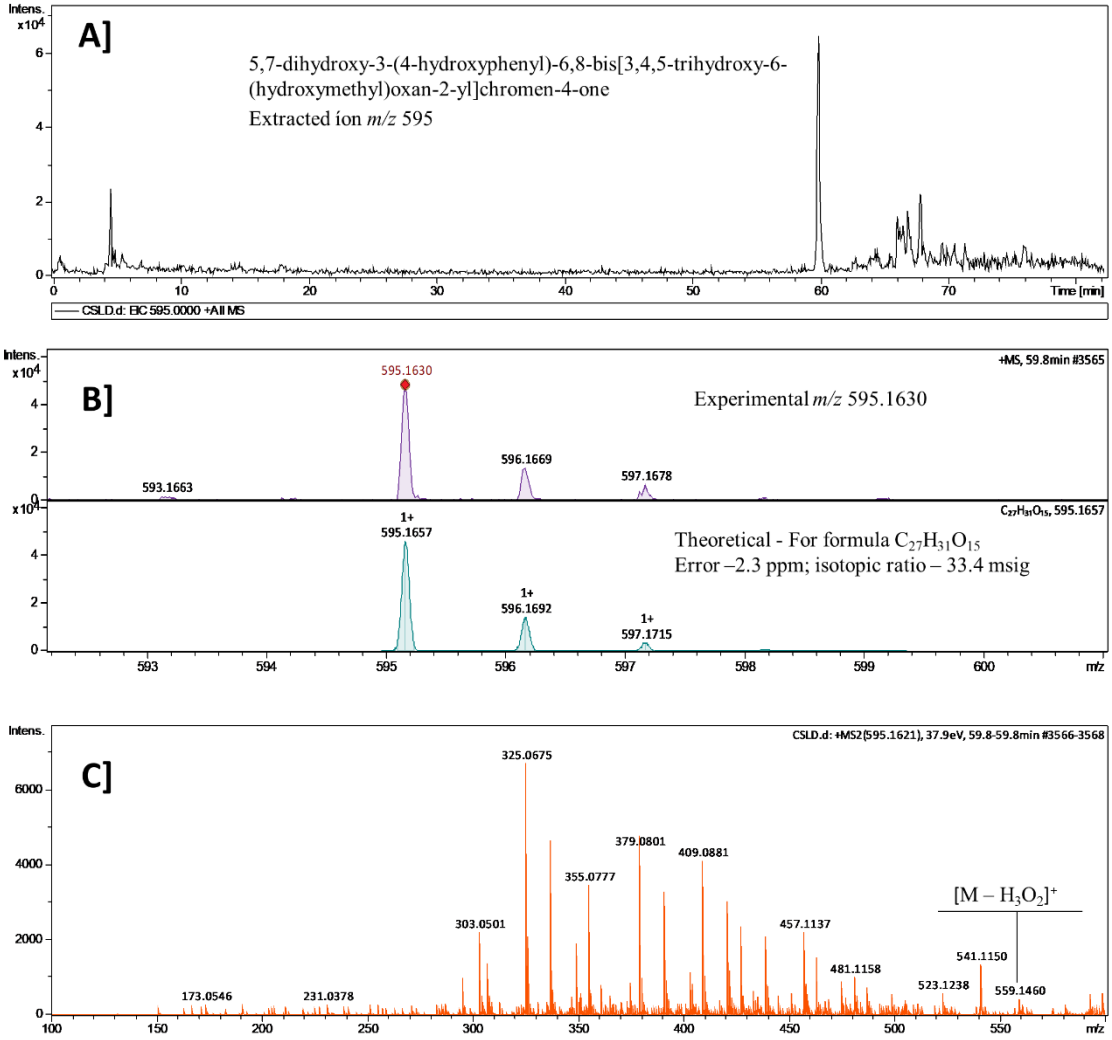
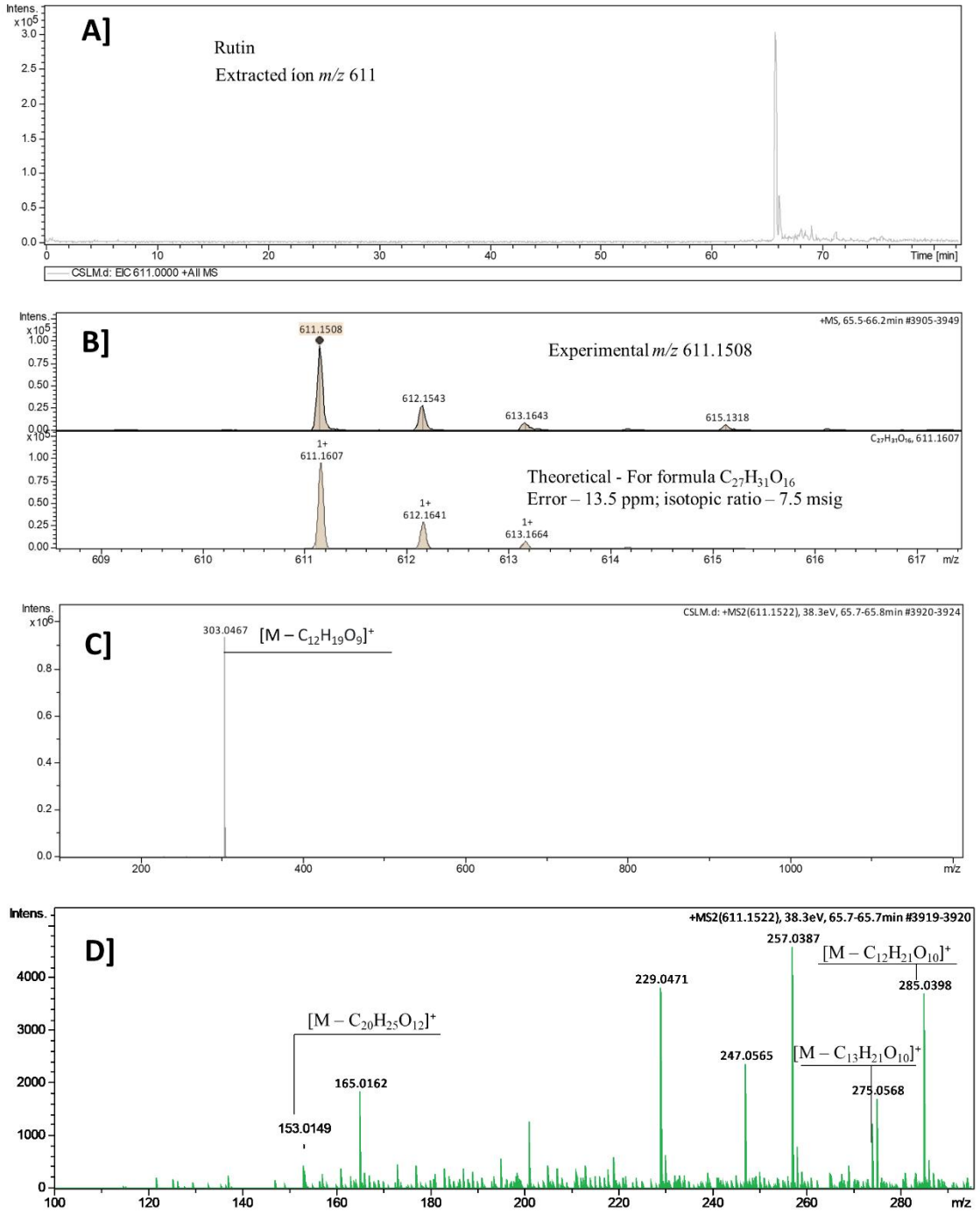


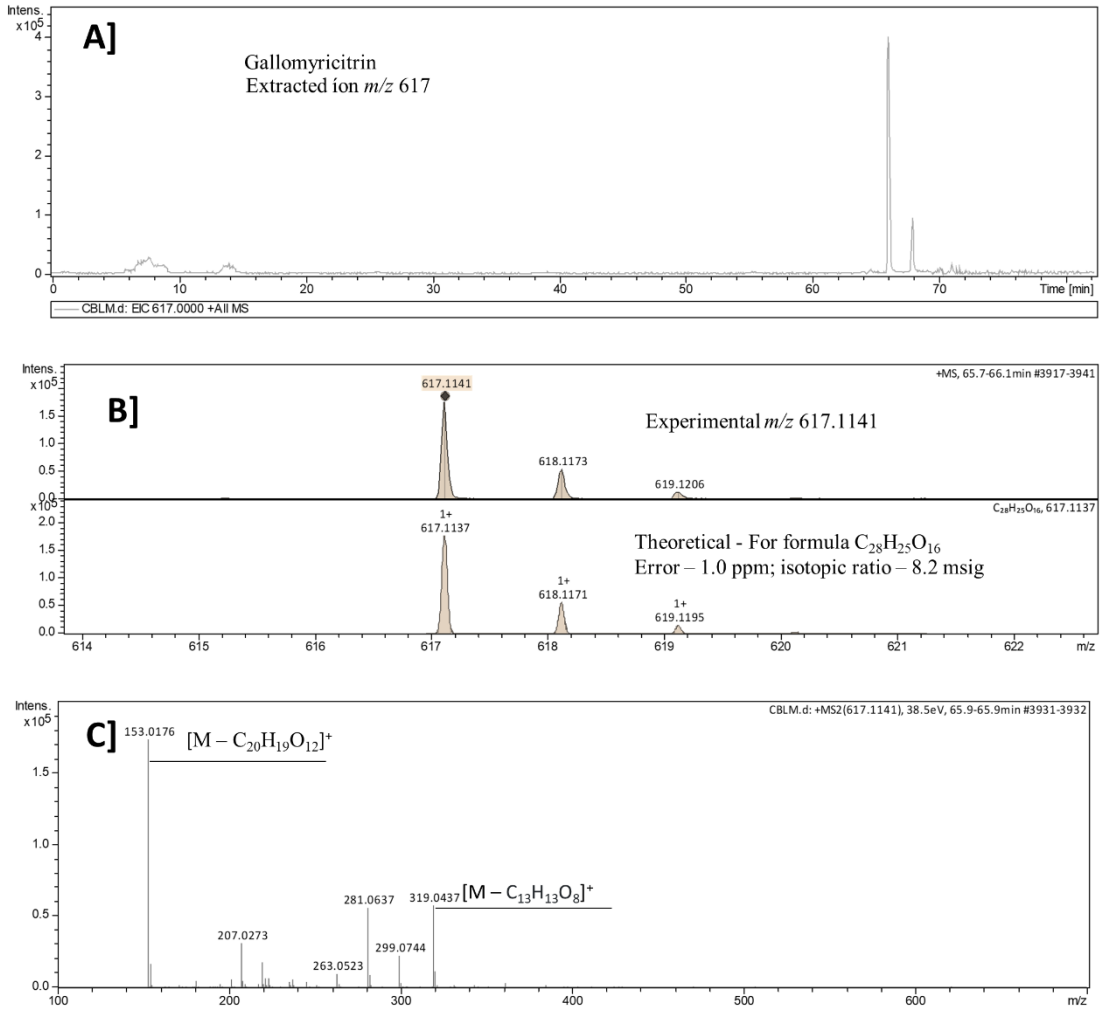
Figure 122: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 595.1630 in MS-MS mode HRESIMS spectrum.



137

138 Figure 133: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
139 which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion *m/z* 611.1508
140 in MS-MS mode HRESIMS spectrum.

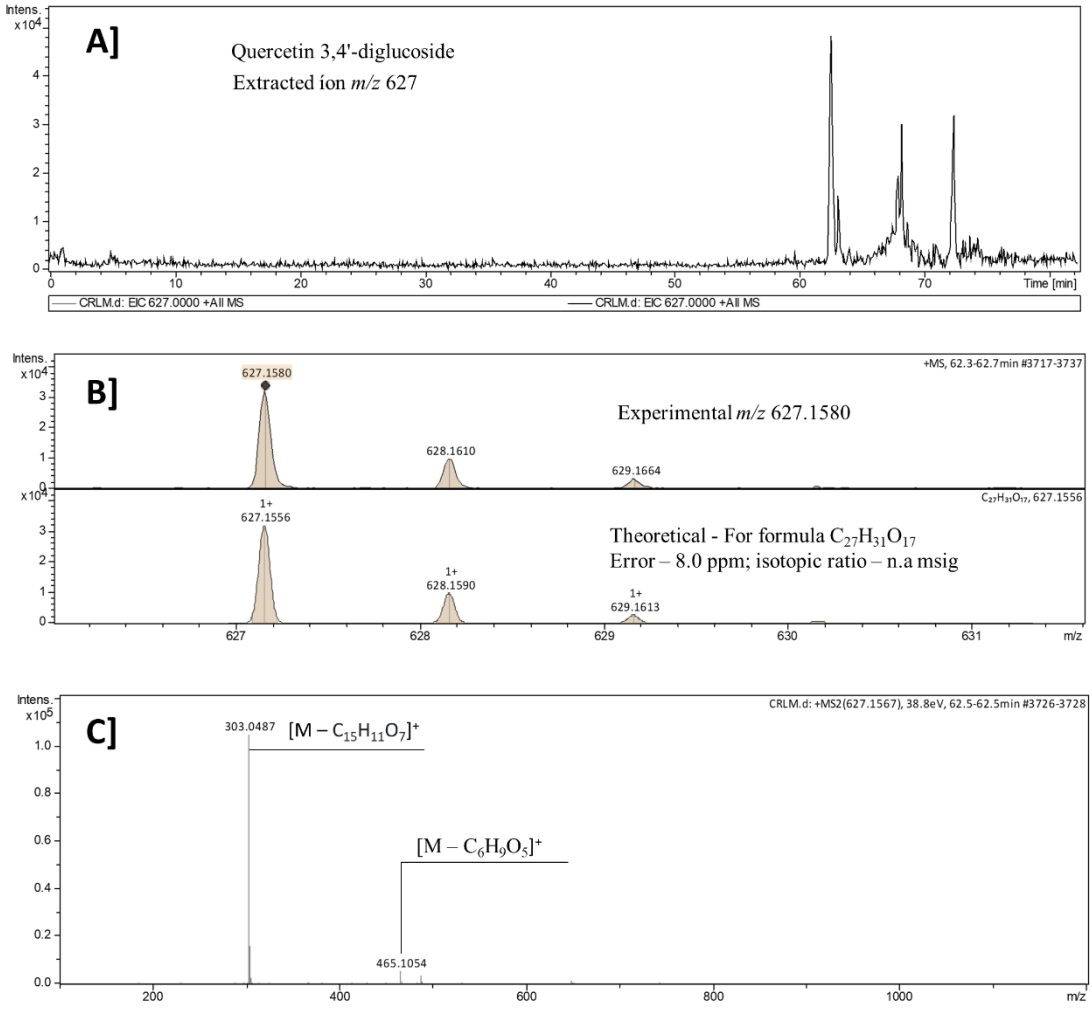
141



142

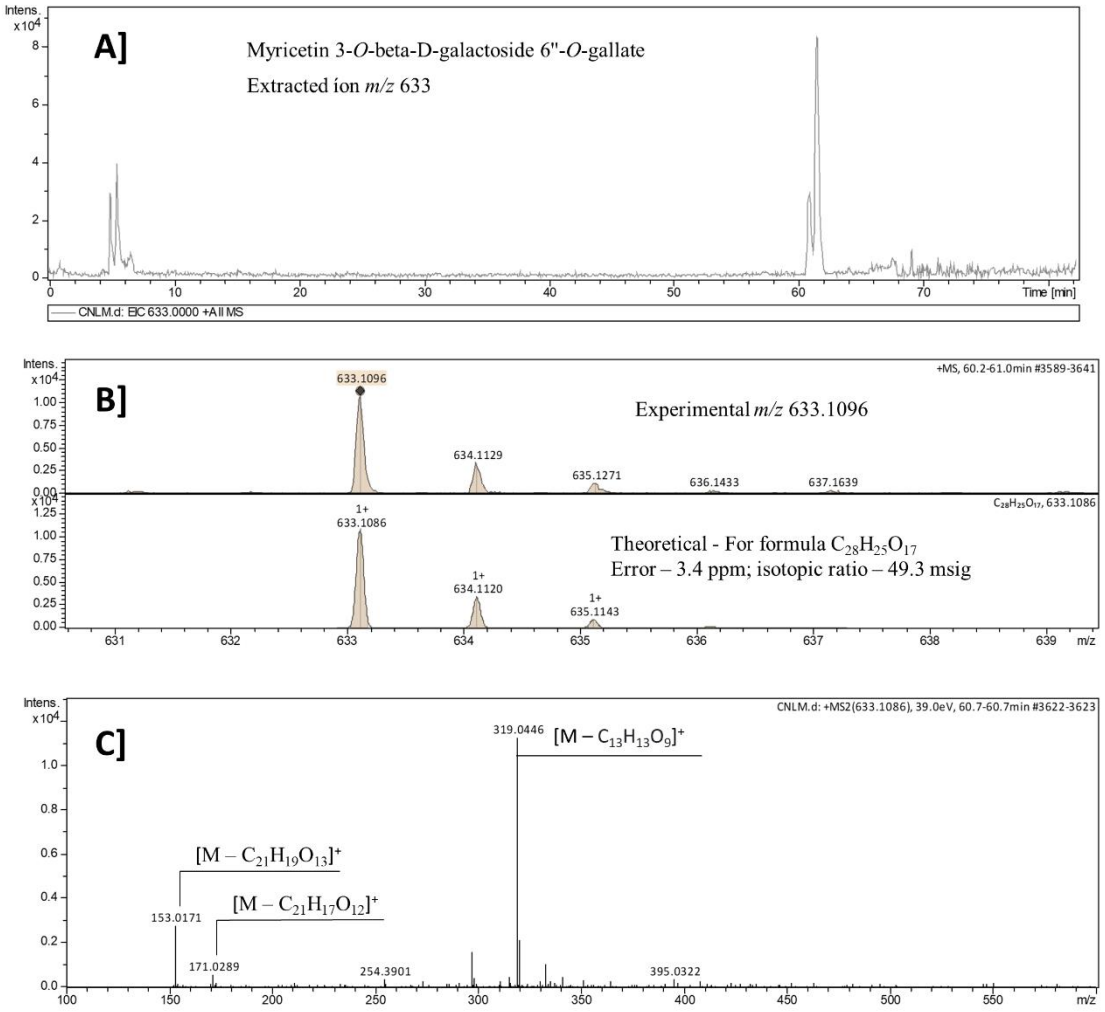
143 Figure 24: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
144 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 617.1141 in MS-
145 MS mode HRESIMS spectrum.

146



147

148 Figure 25: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
149 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 627.1580 in MS-
150 MS mode HRESIMS spectrum.



151

152 Figure 146: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
153 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion *m/z* 633.1096 in MS-
154 MS mode HRESIMS spectrum.

Chemical Composition, Antioxidant and Anti-AGEs Activities in four species of the Connaraceae Family

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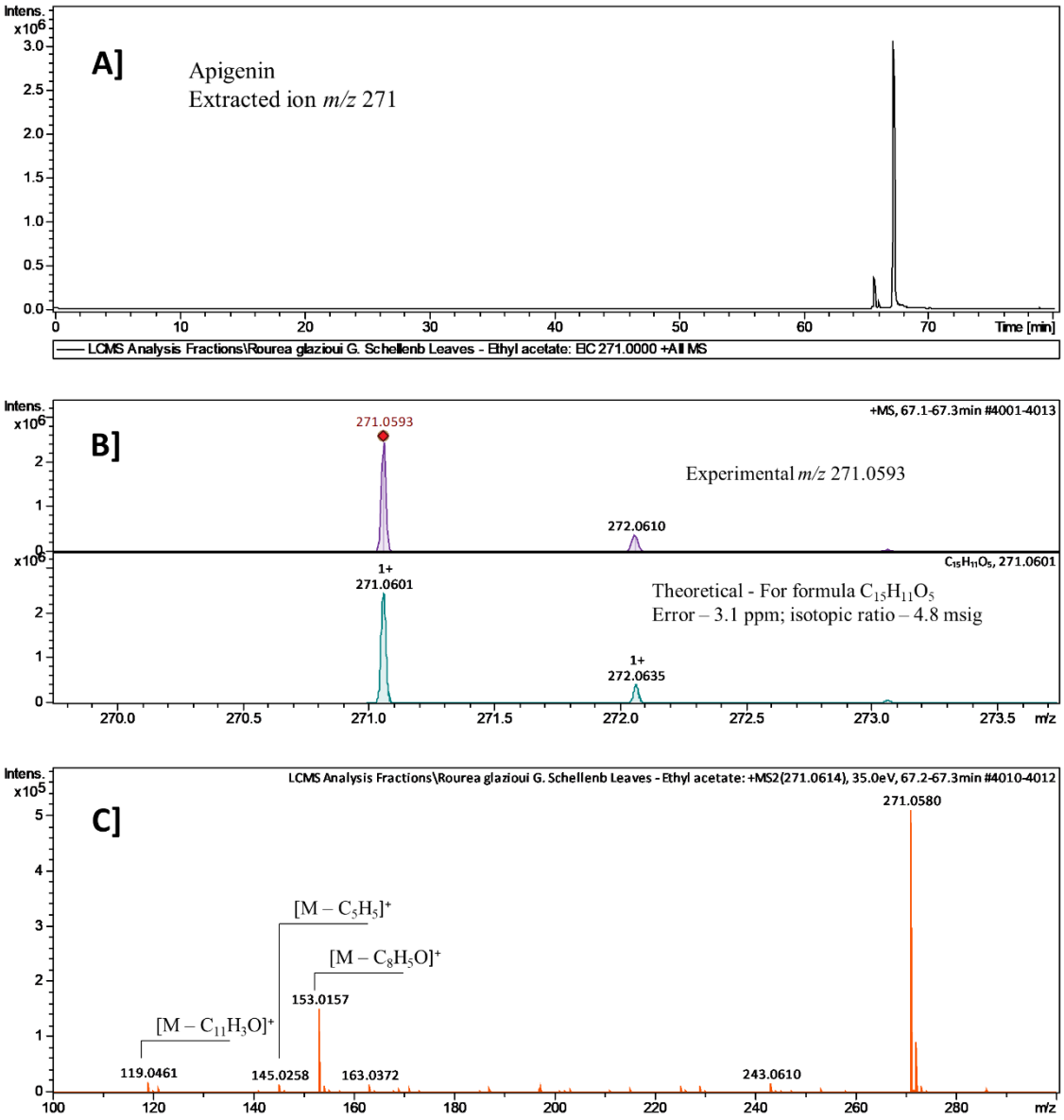
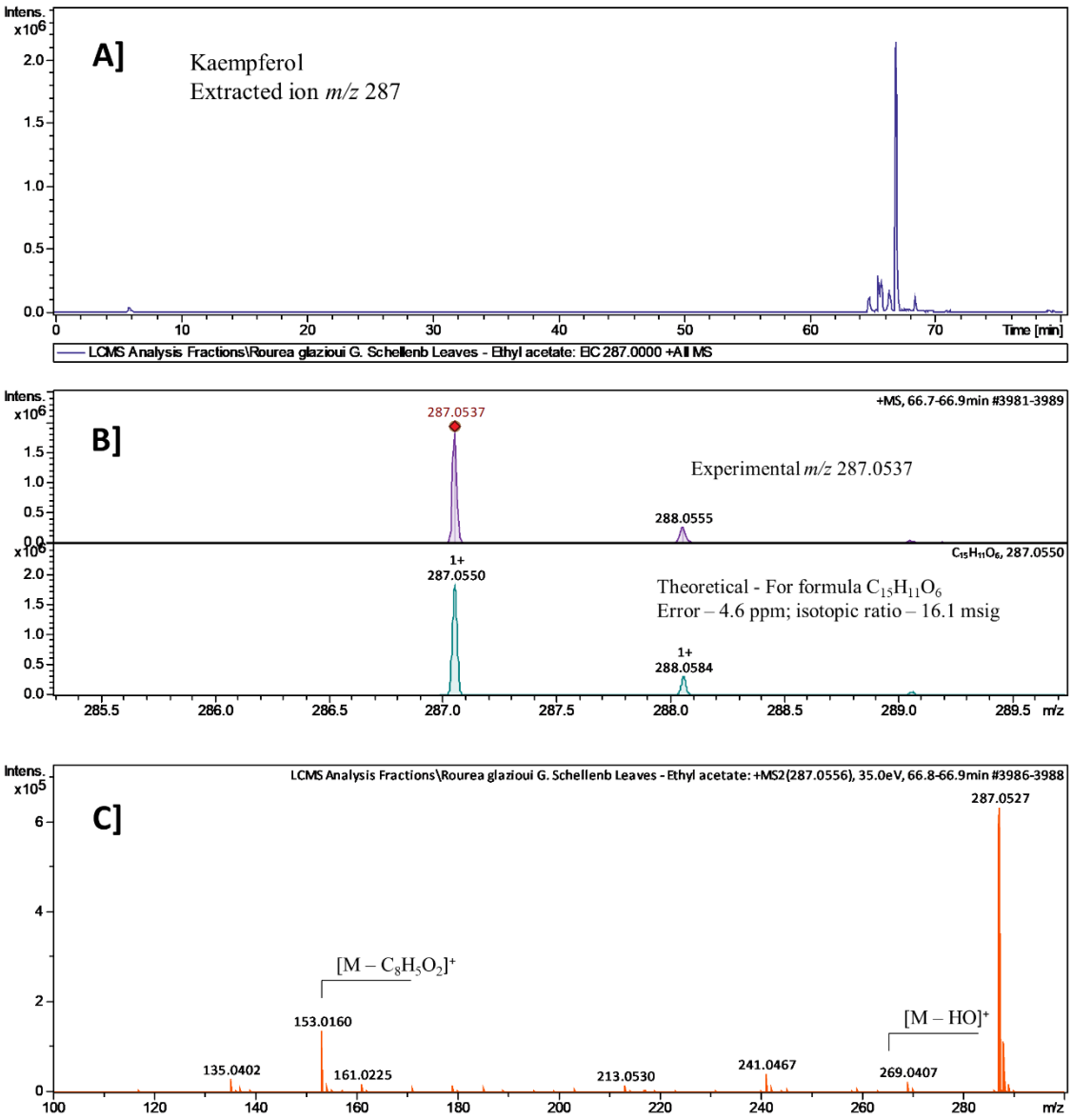
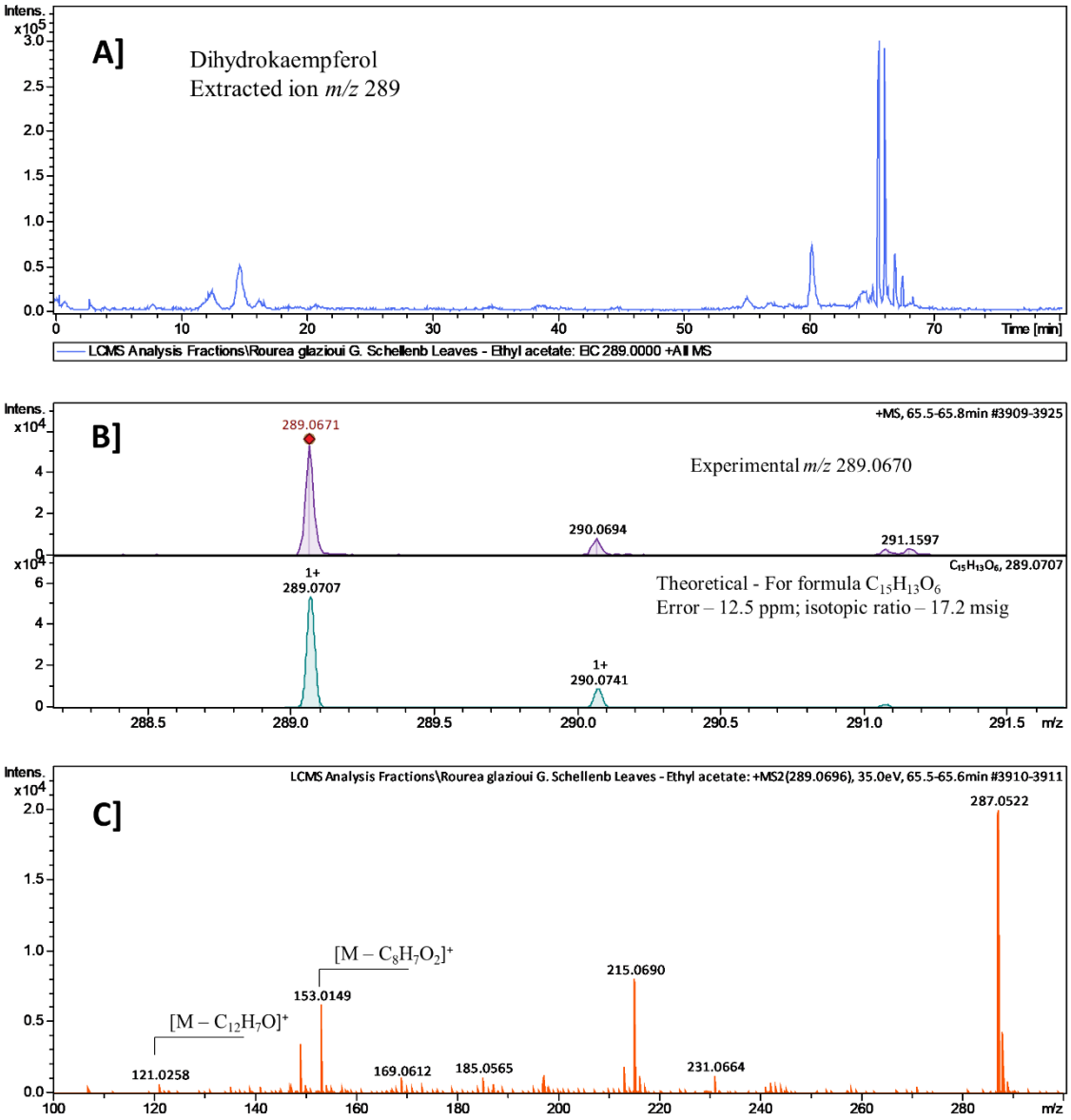


Figure 1: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 271.0593 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.



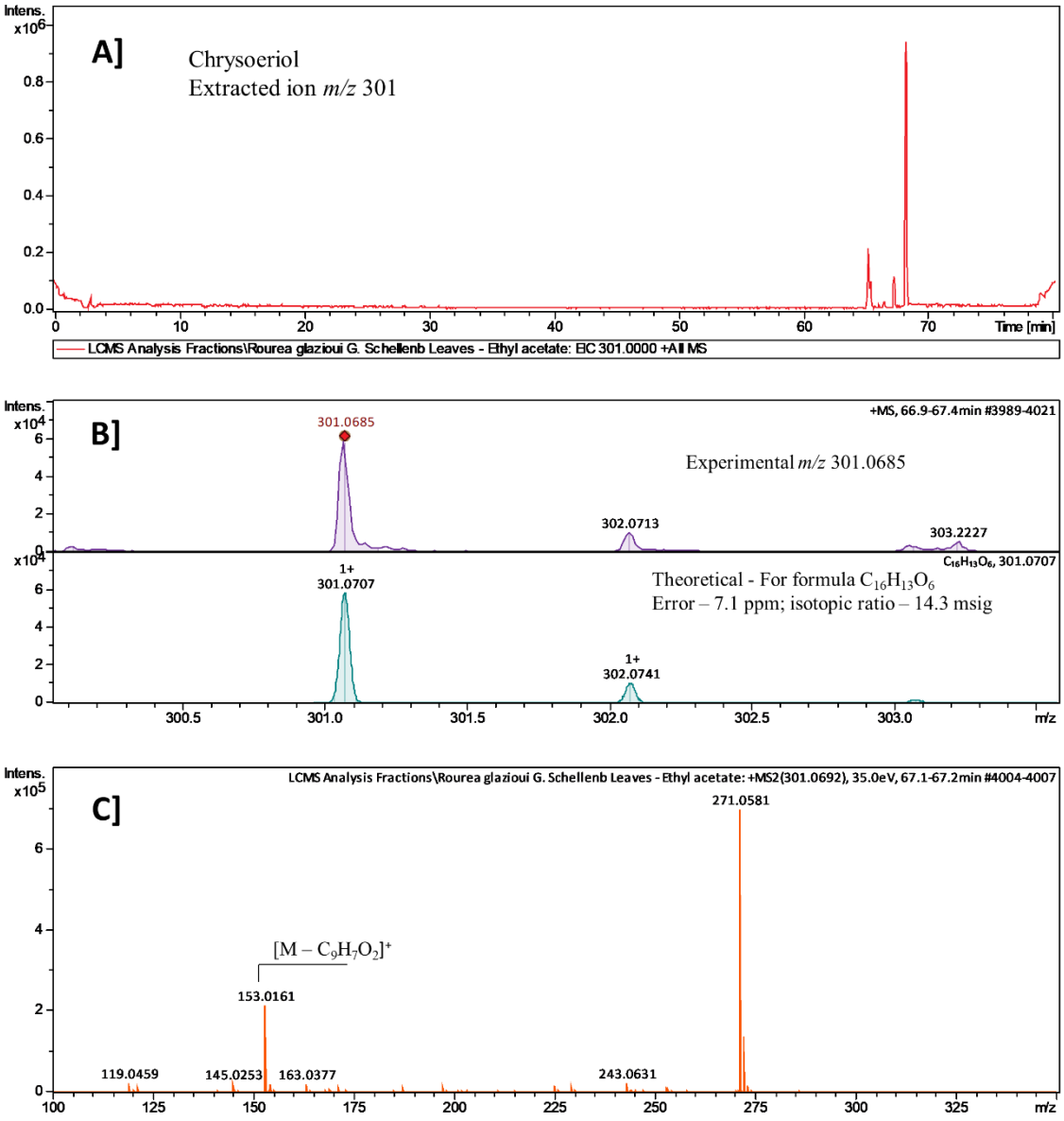
31

32 Figure 2: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
33 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 287.0537 in MS-
34 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
35 search for isotopic masses at ChemCalc.



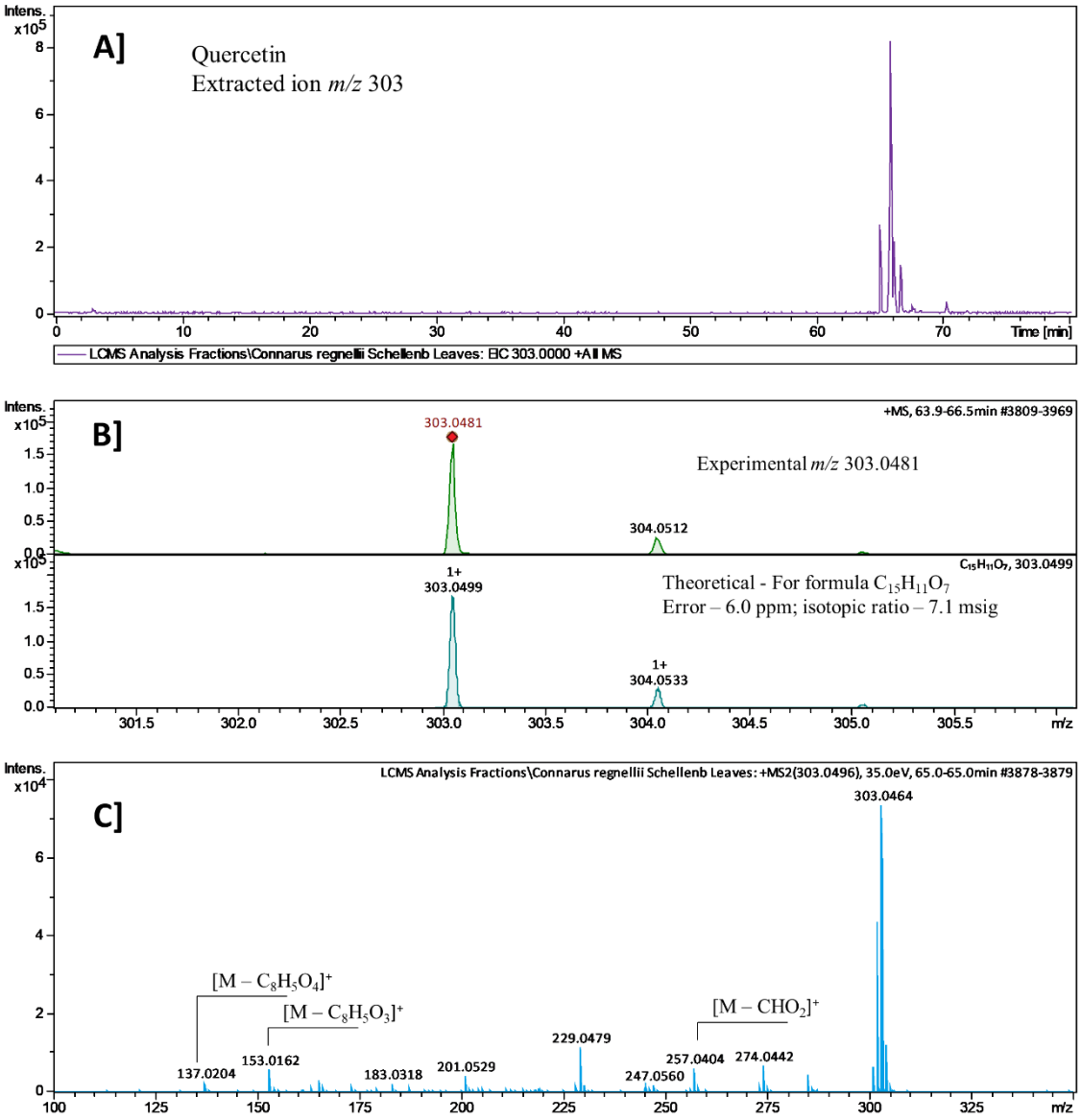
36

37 Figure 3: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
38 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 289.0670 in MS-
39 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
40 search for isotopic masses at ChemCalc.



41

42 Figure 4: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
43 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 301.0685 in MS-
44 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
45 search for isotopic masses at ChemCalc.



46

47 Figure 5: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
48 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 303.0481 in MS-
49 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
50 search for isotopic masses at ChemCalc.

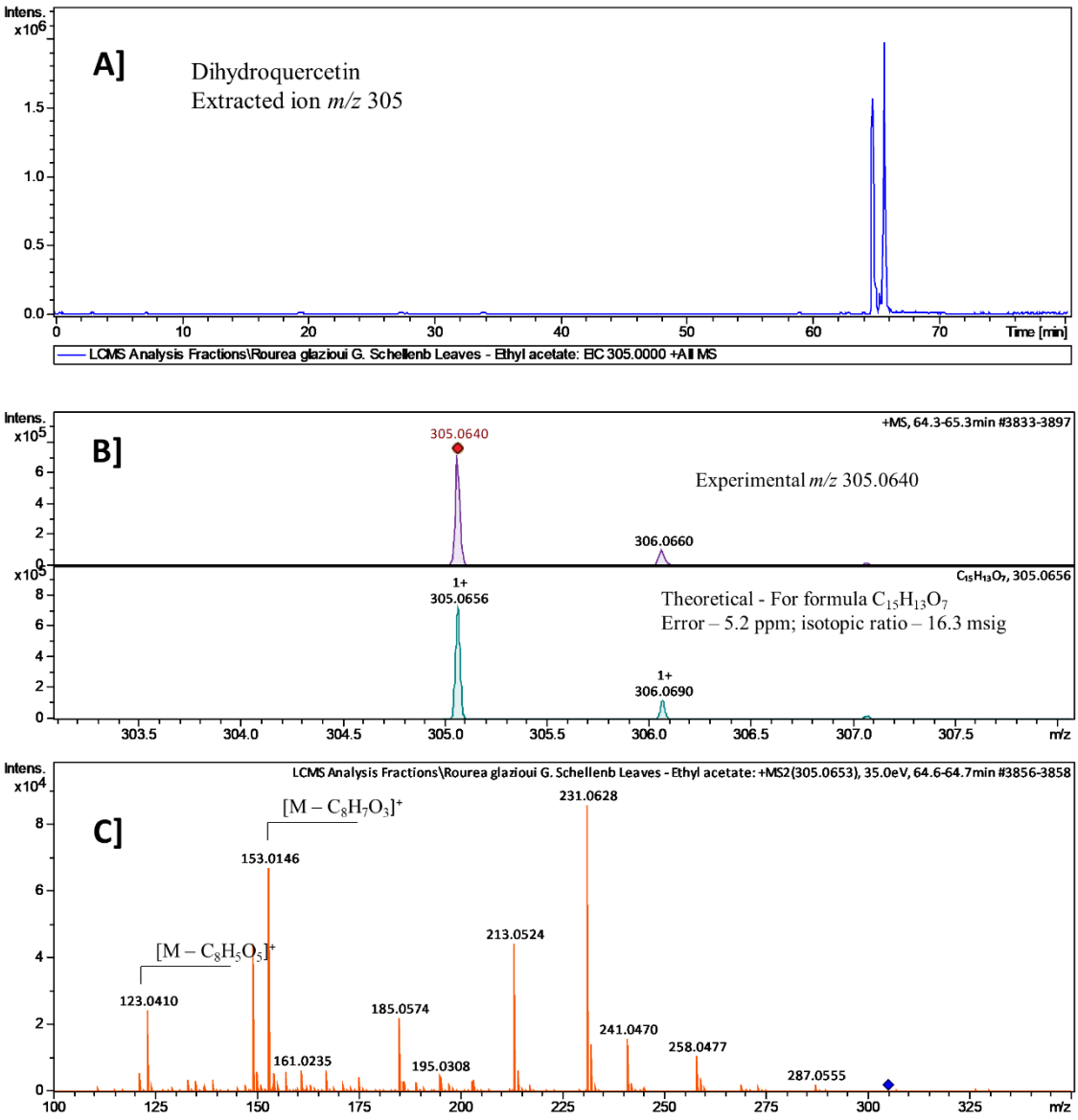


Figure 6: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 305.0640 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.

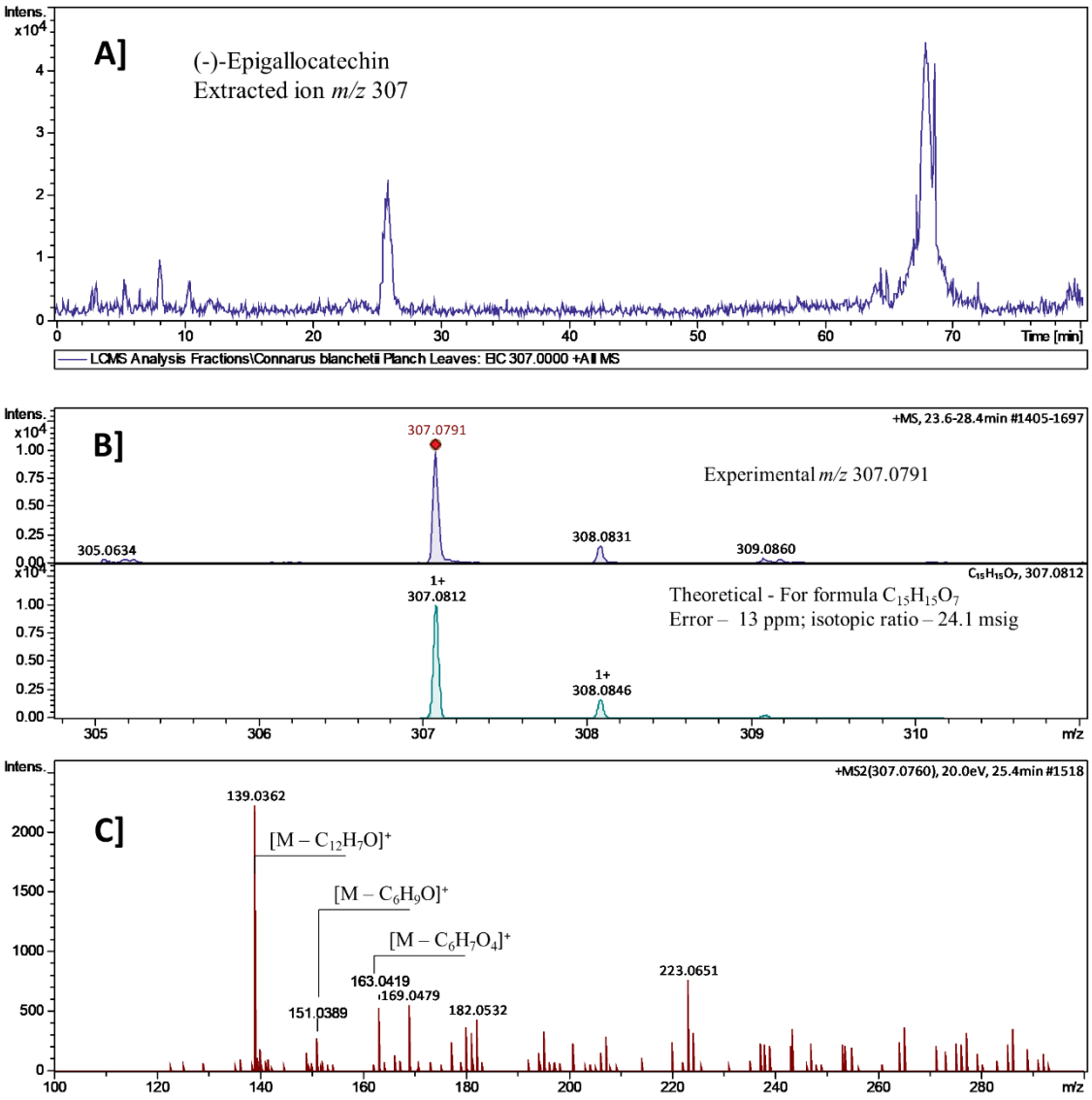


Figure 7: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 307.0791 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.

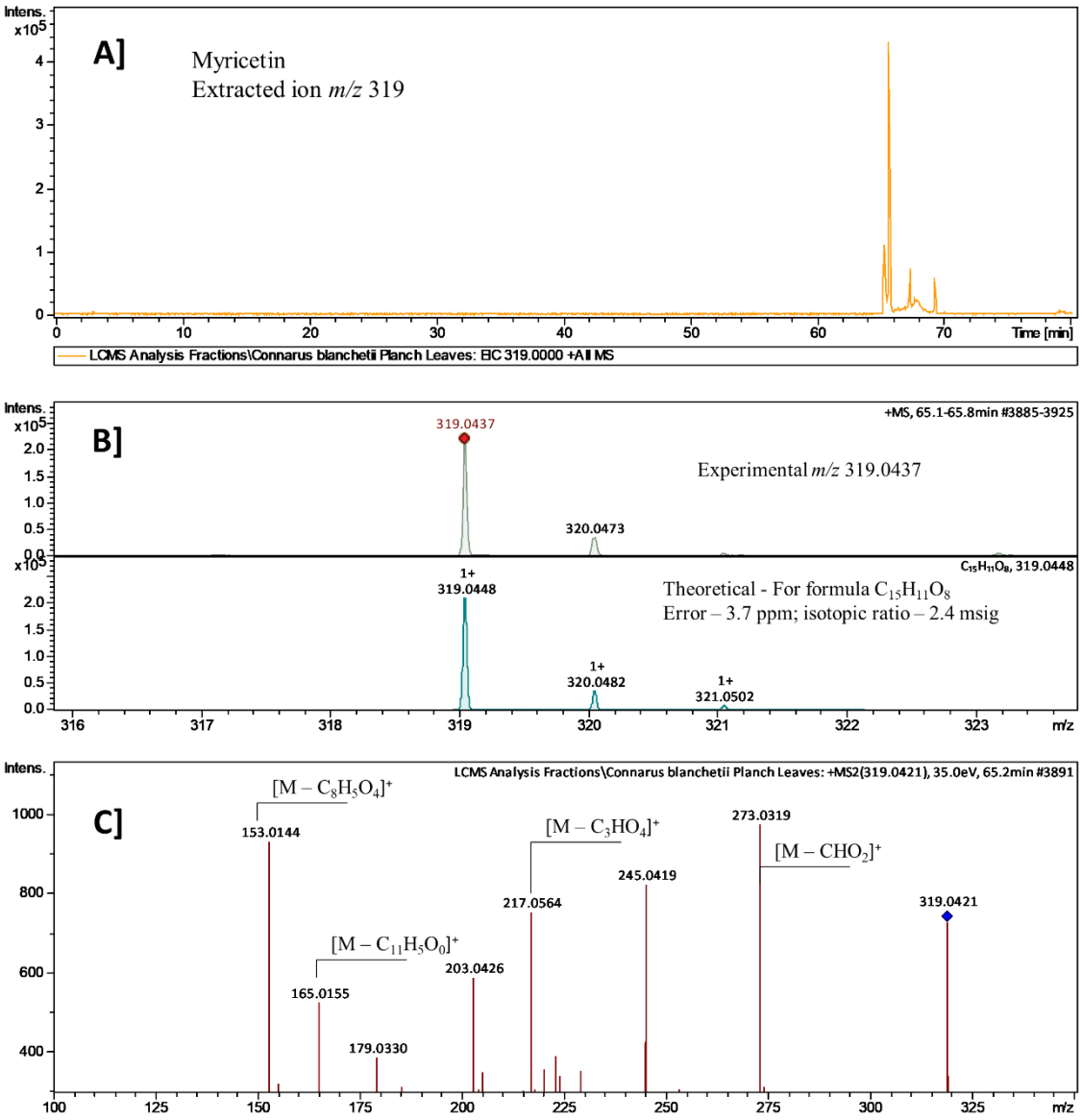
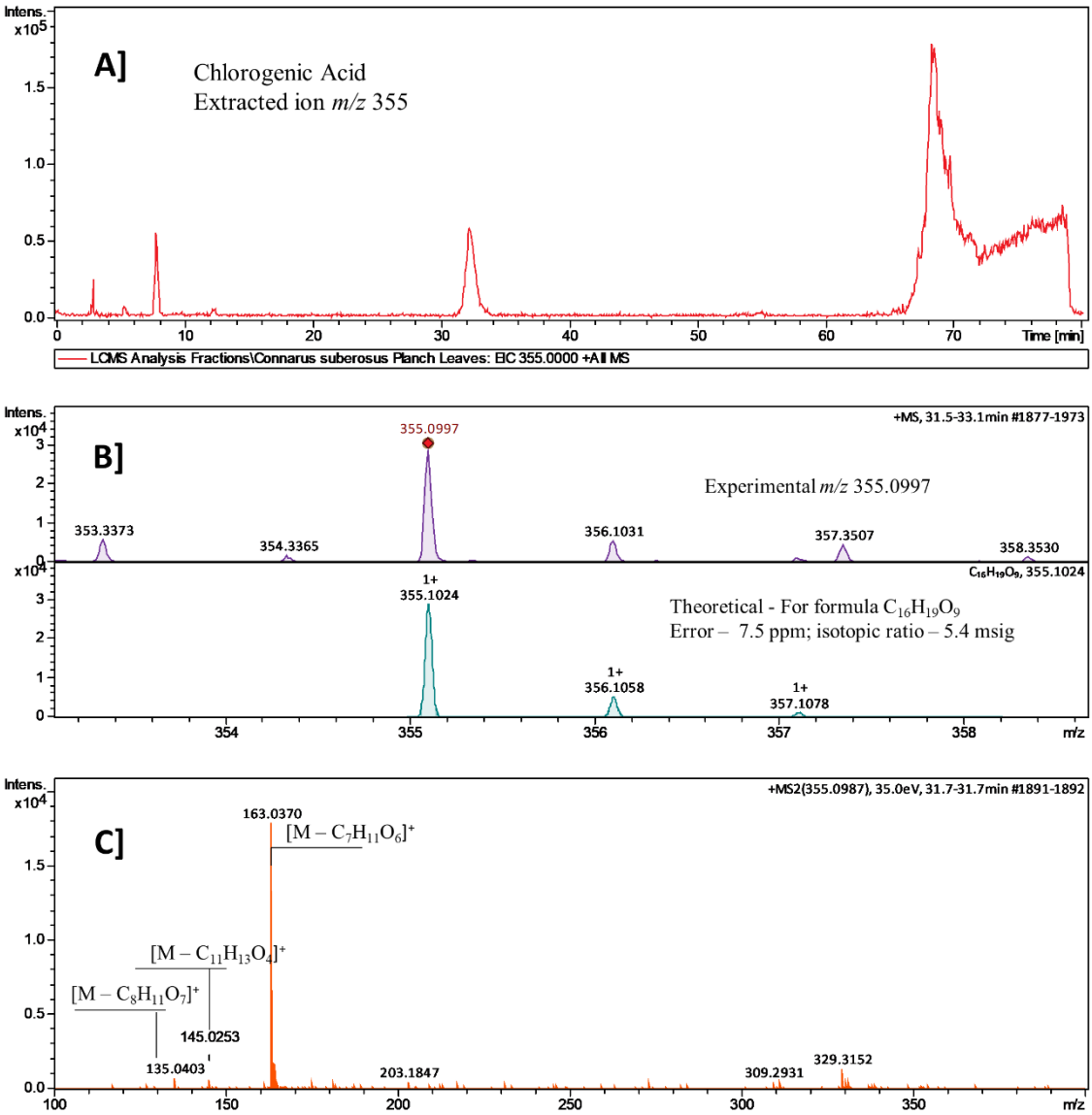
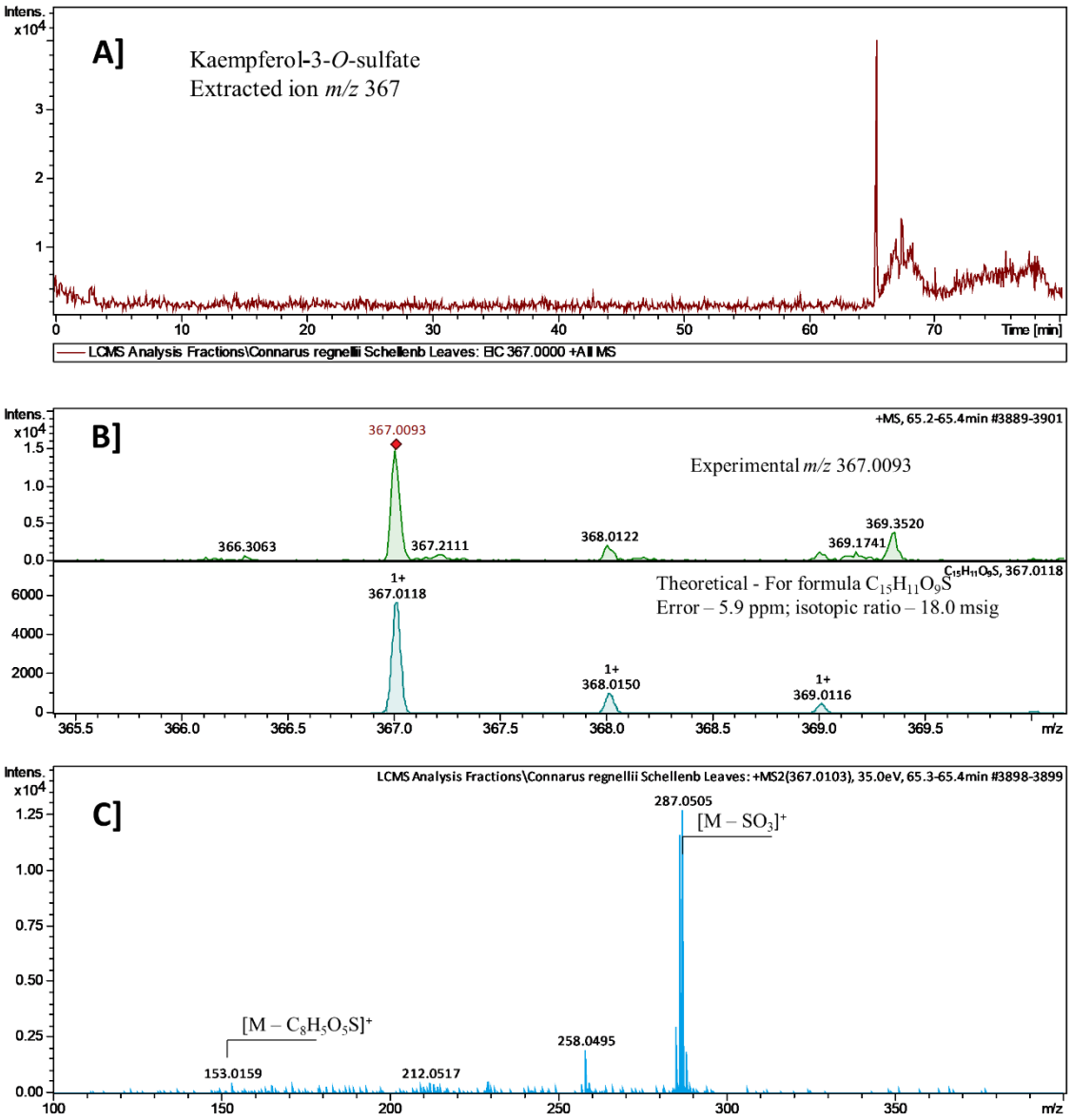


Figure 8: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 319.0437 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.



66

67 Figure 9: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
68 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 355.0997 in MS-
69 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
70 search for isotopic masses at ChemCalc.



71

72 Figure 10: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
73 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 367.0093 in MS-
74 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
75 search for isotopic masses at ChemCalc.

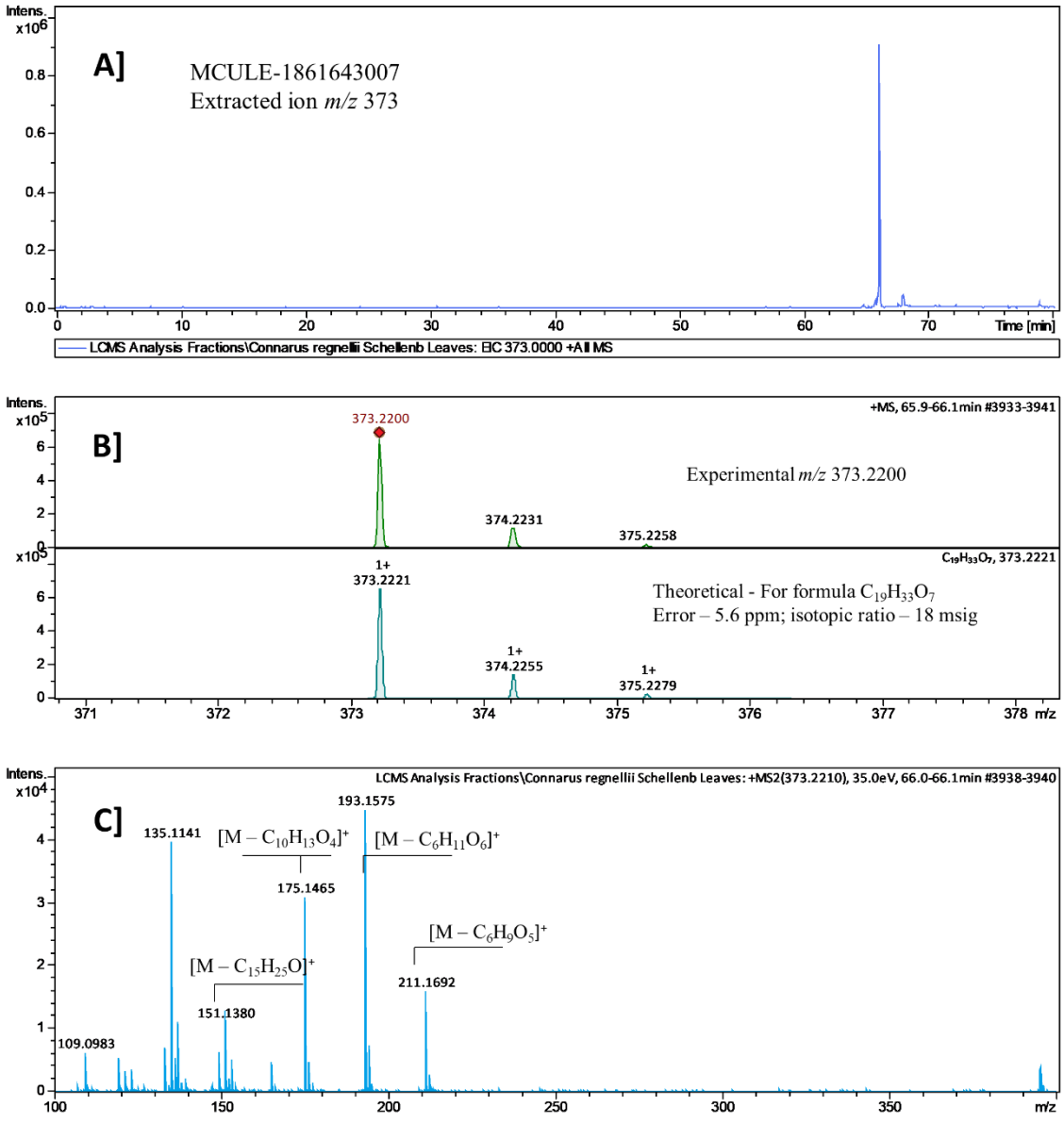
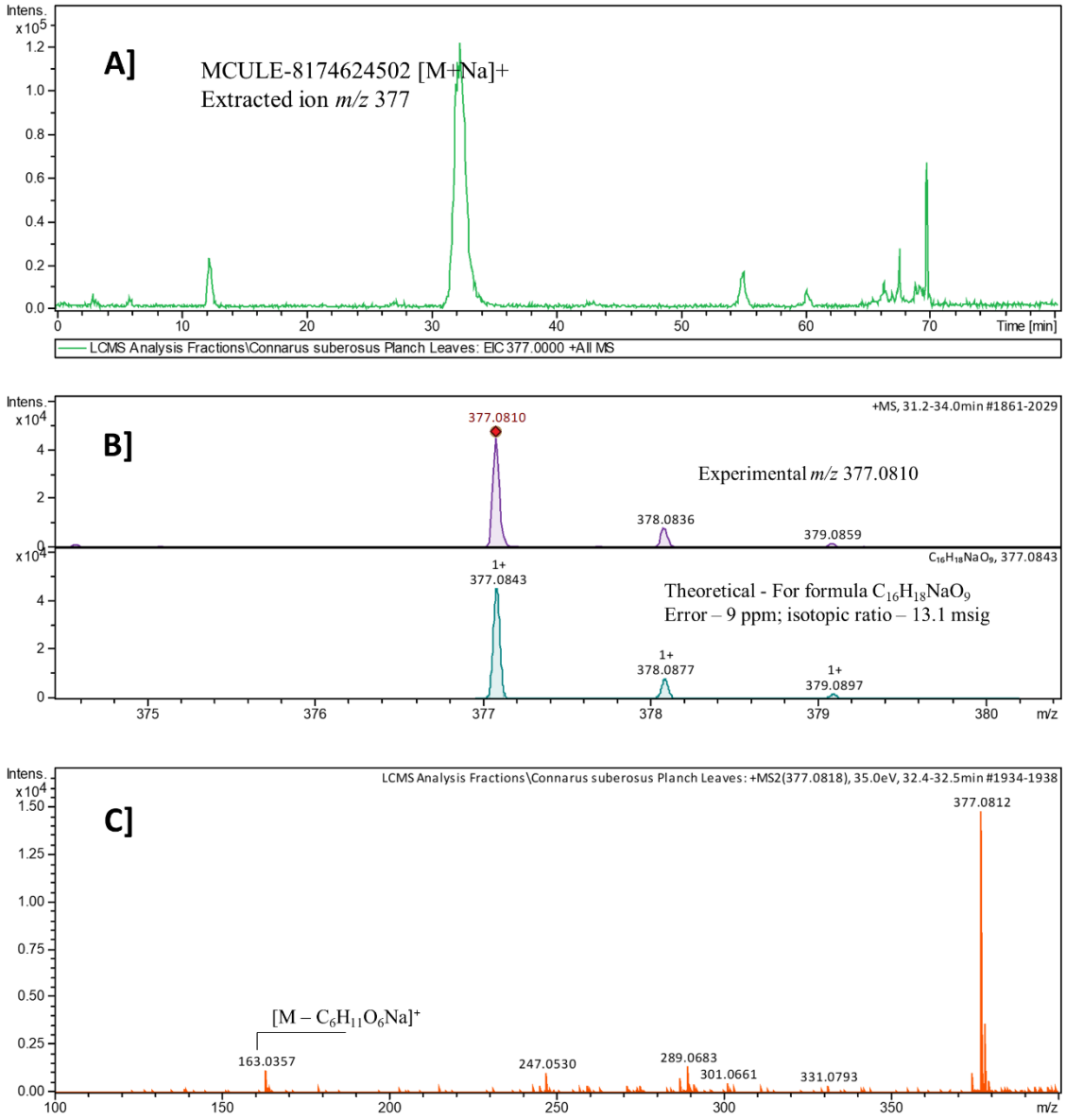
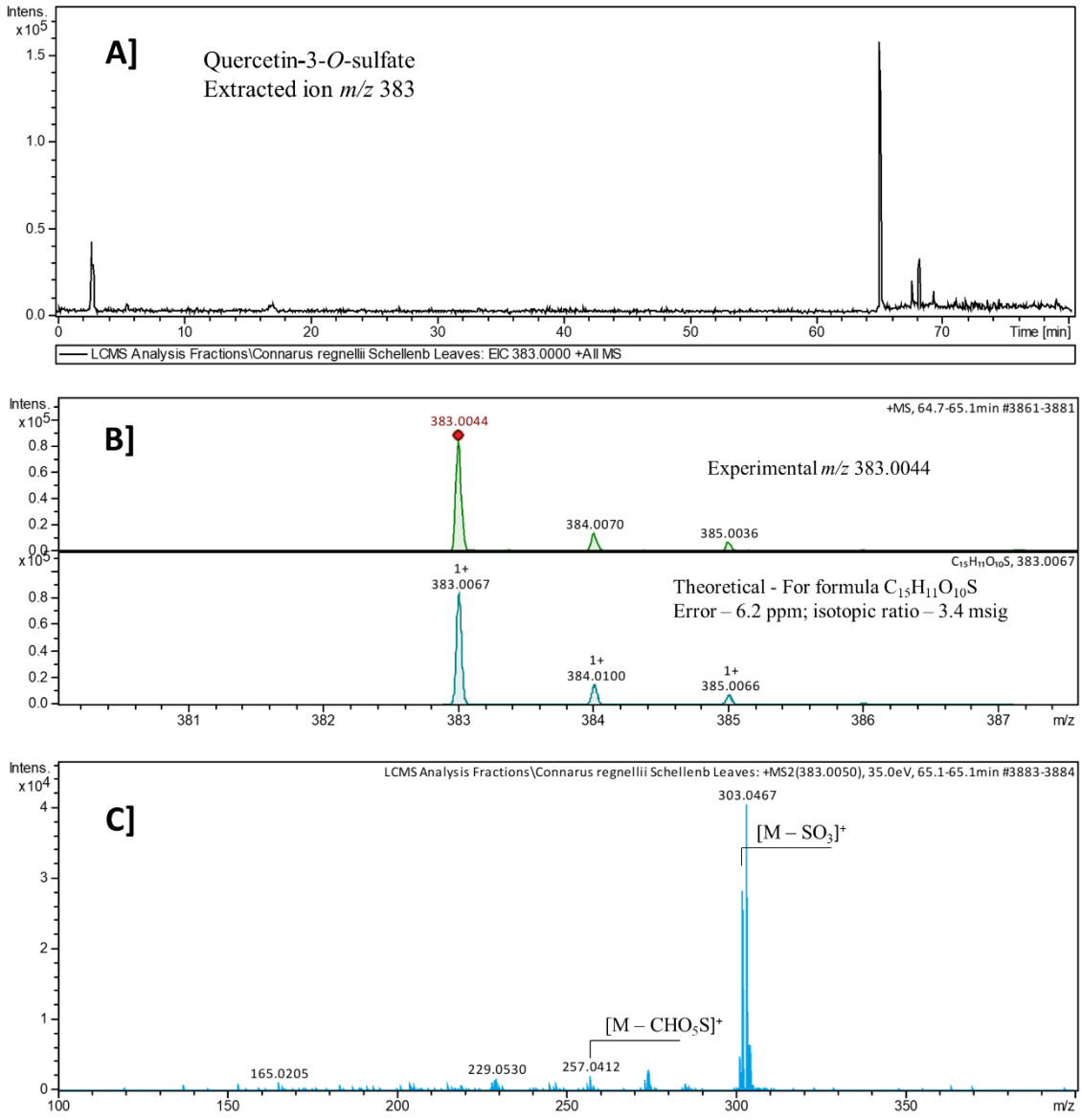


Figure 11: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 373.2200 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.



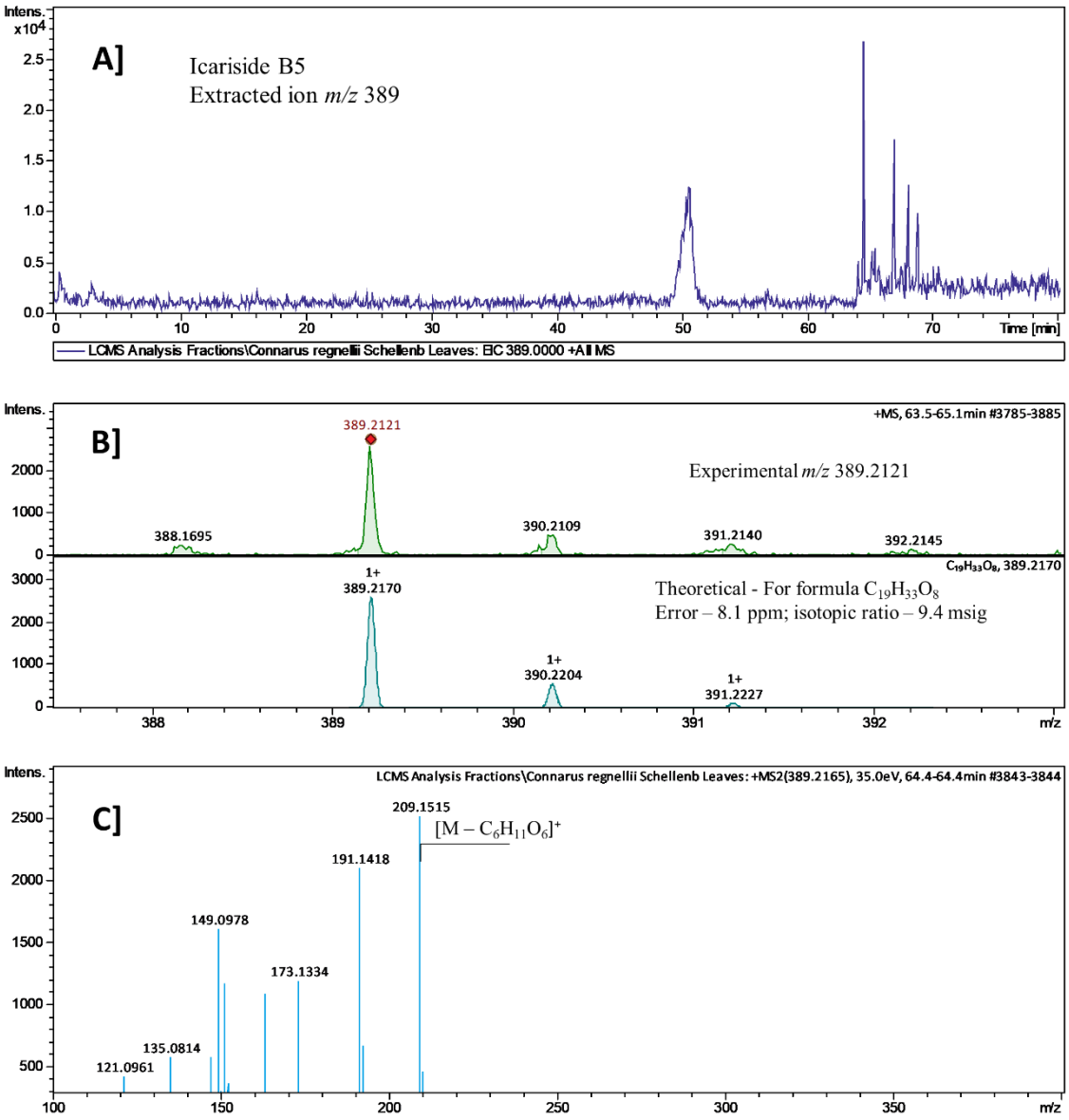
81

82 Figure 12: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
83 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 377.0810 in MS-
84 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
85 search for isotopic masses at ChemCalc.



86

87 Figure 13: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
88 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 383.0044 in MS-
89 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
90 search for isotopic masses at ChemCalc.



91

92 Figure 14: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
93 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 389.2121 in MS-
94 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
95 search for isotopic masses at ChemCalc.

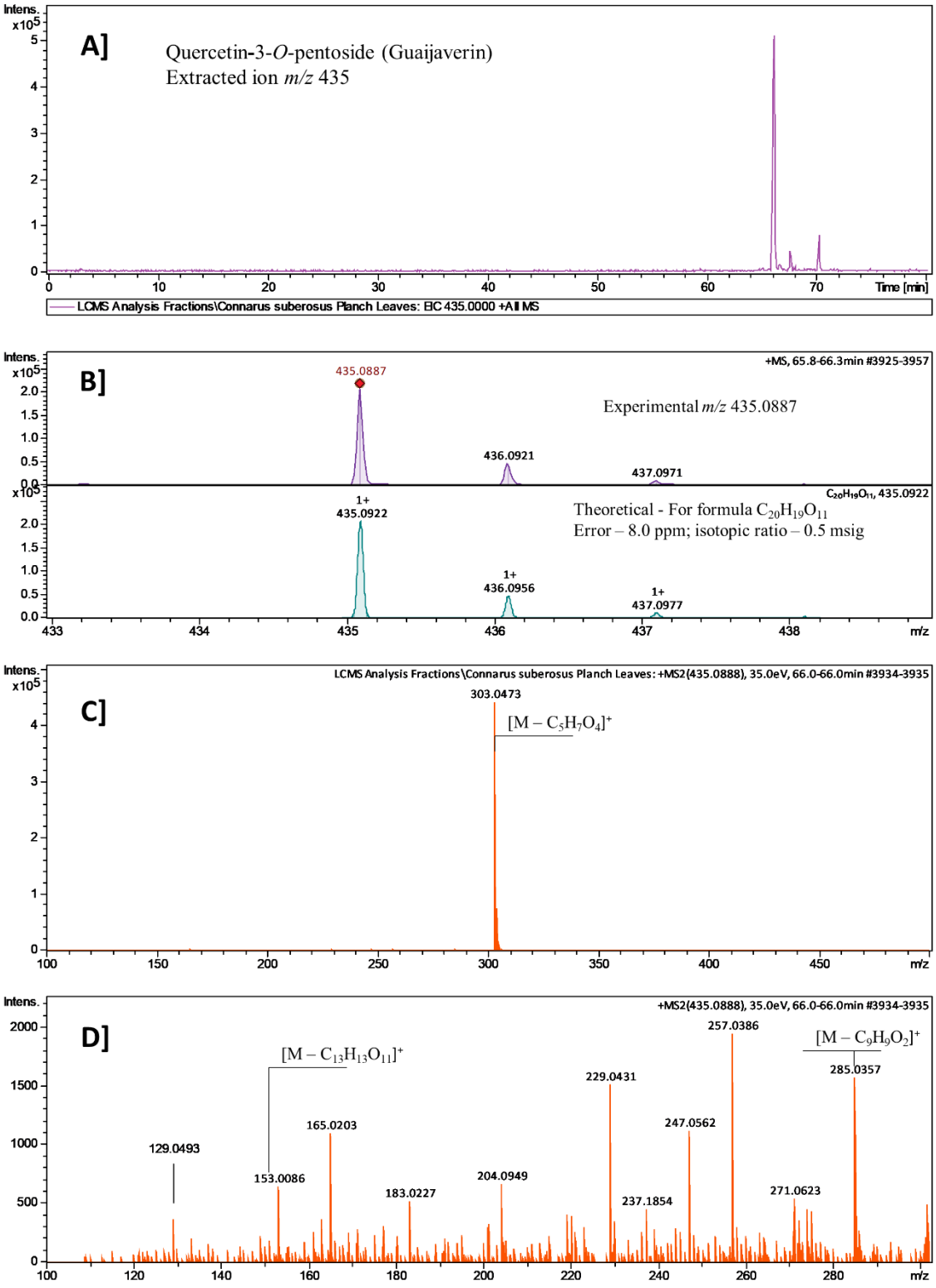
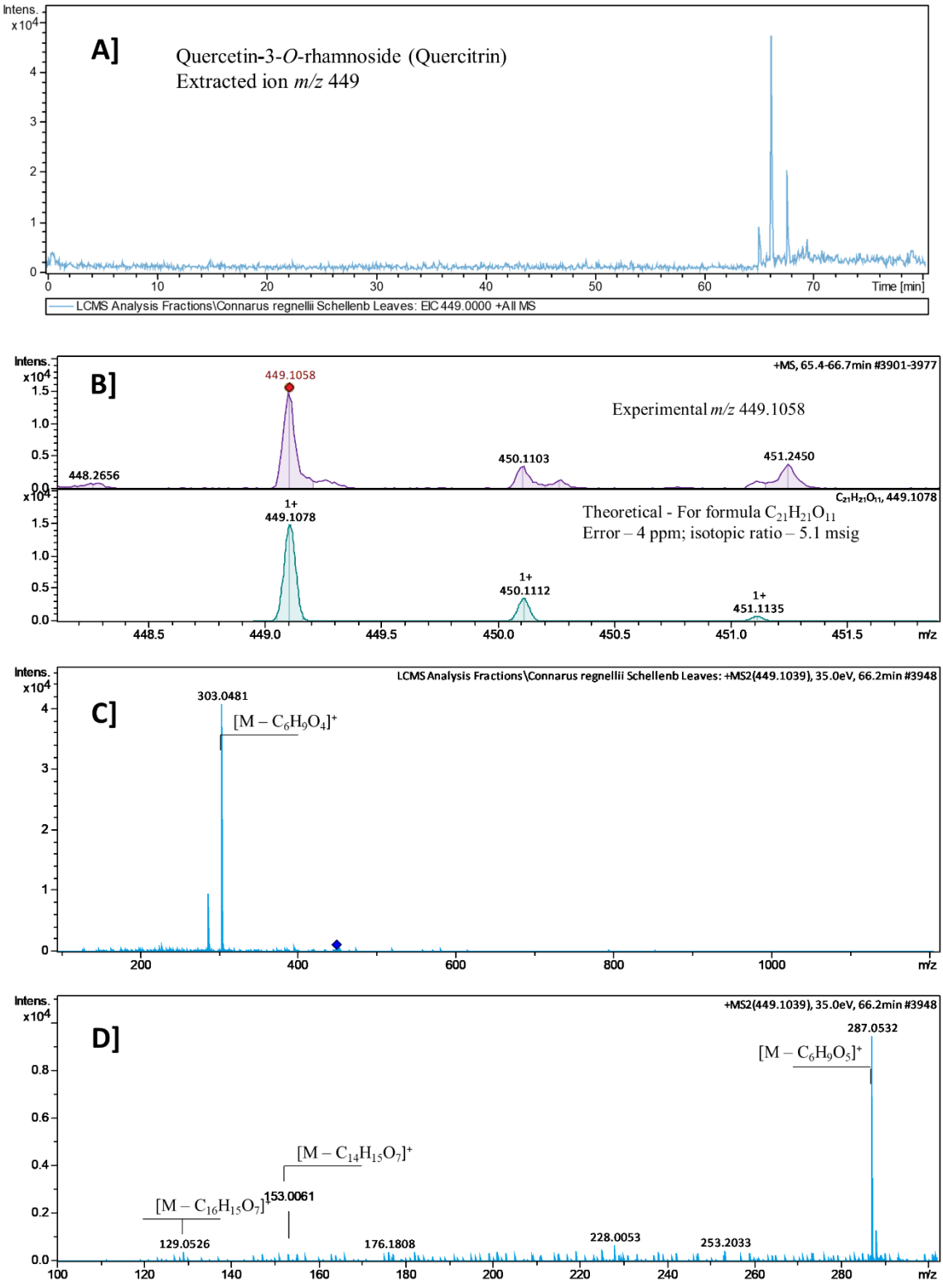
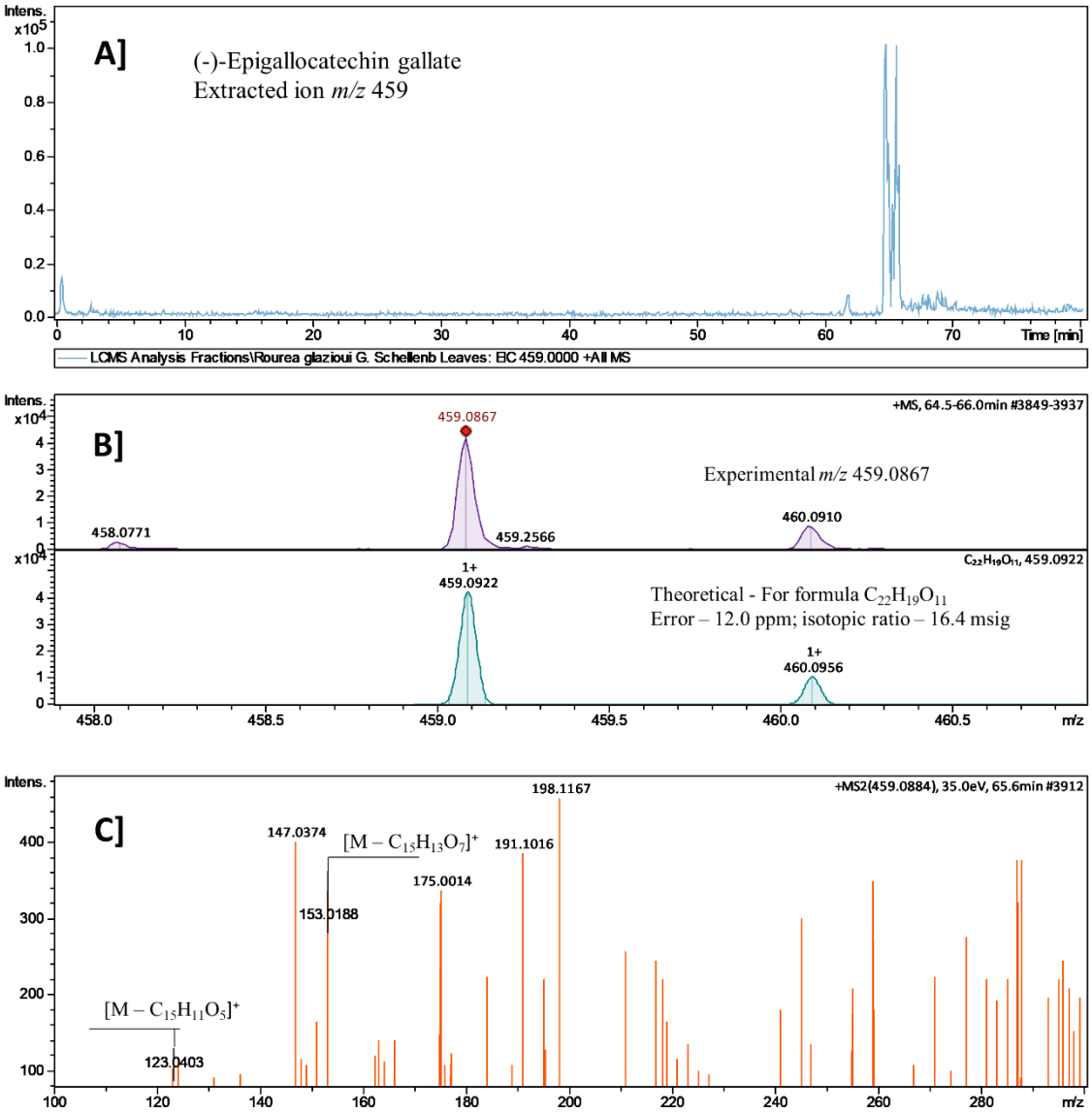


Figure 15: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 435.0887 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.



101

102 Figure 16: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
103 which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 449.1058
104 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID
105 and search for isotopic masses at ChemCalc.



106

107 Figure 17: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
108 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 459.0867 in MS-
109 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
110 search for isotopic masses at ChemCalc.

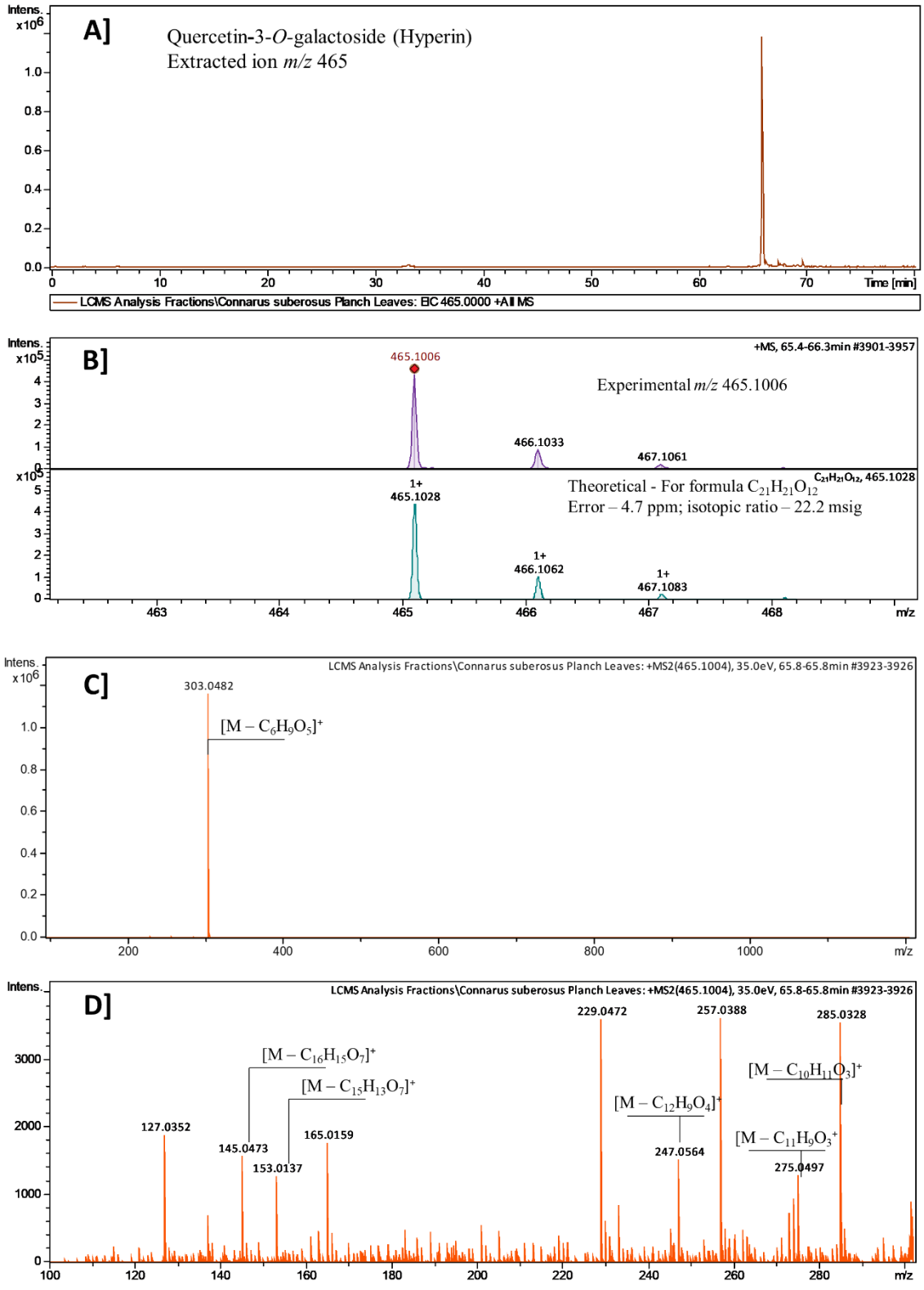


Figure 18: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 465.1006 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.

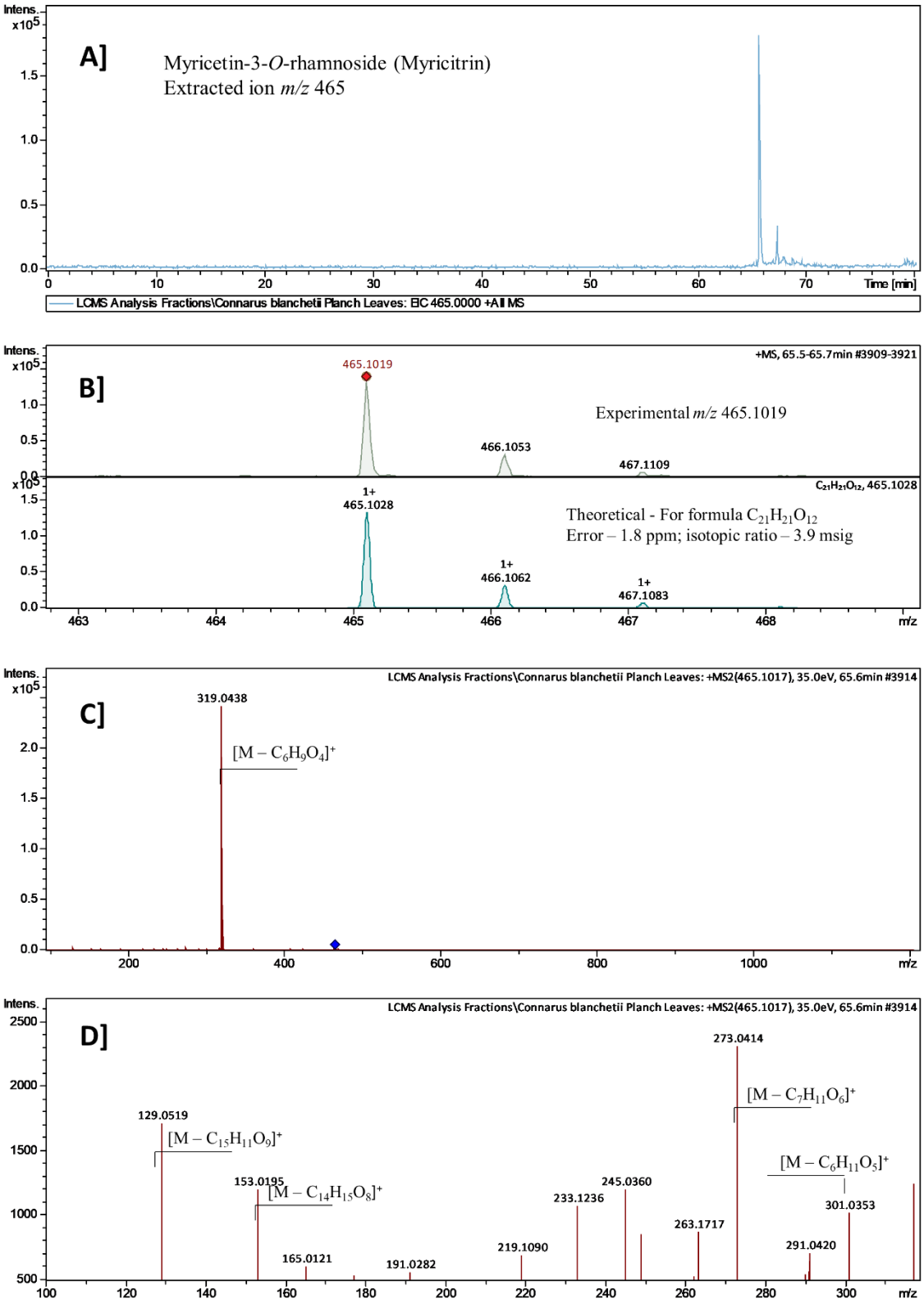
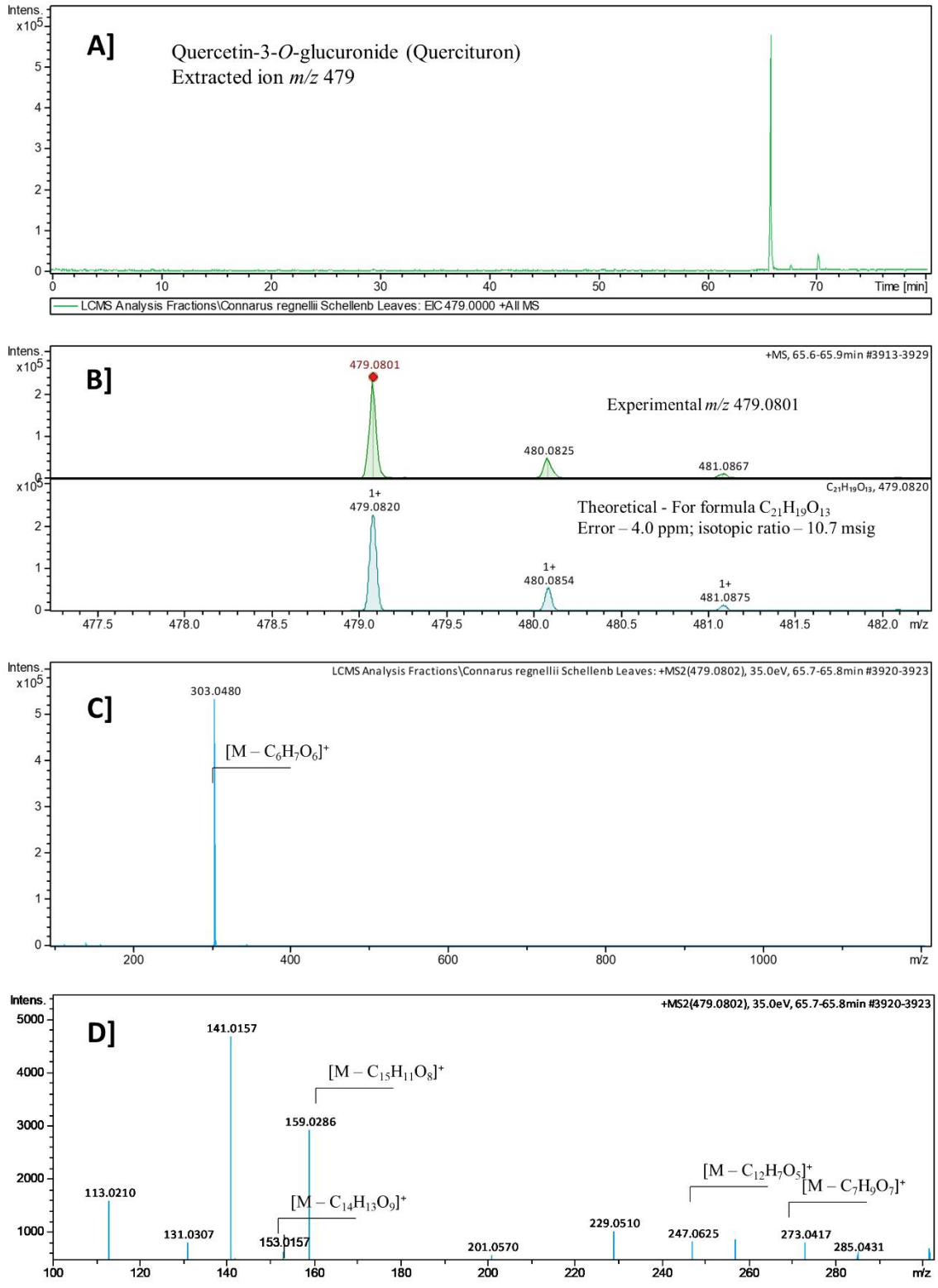
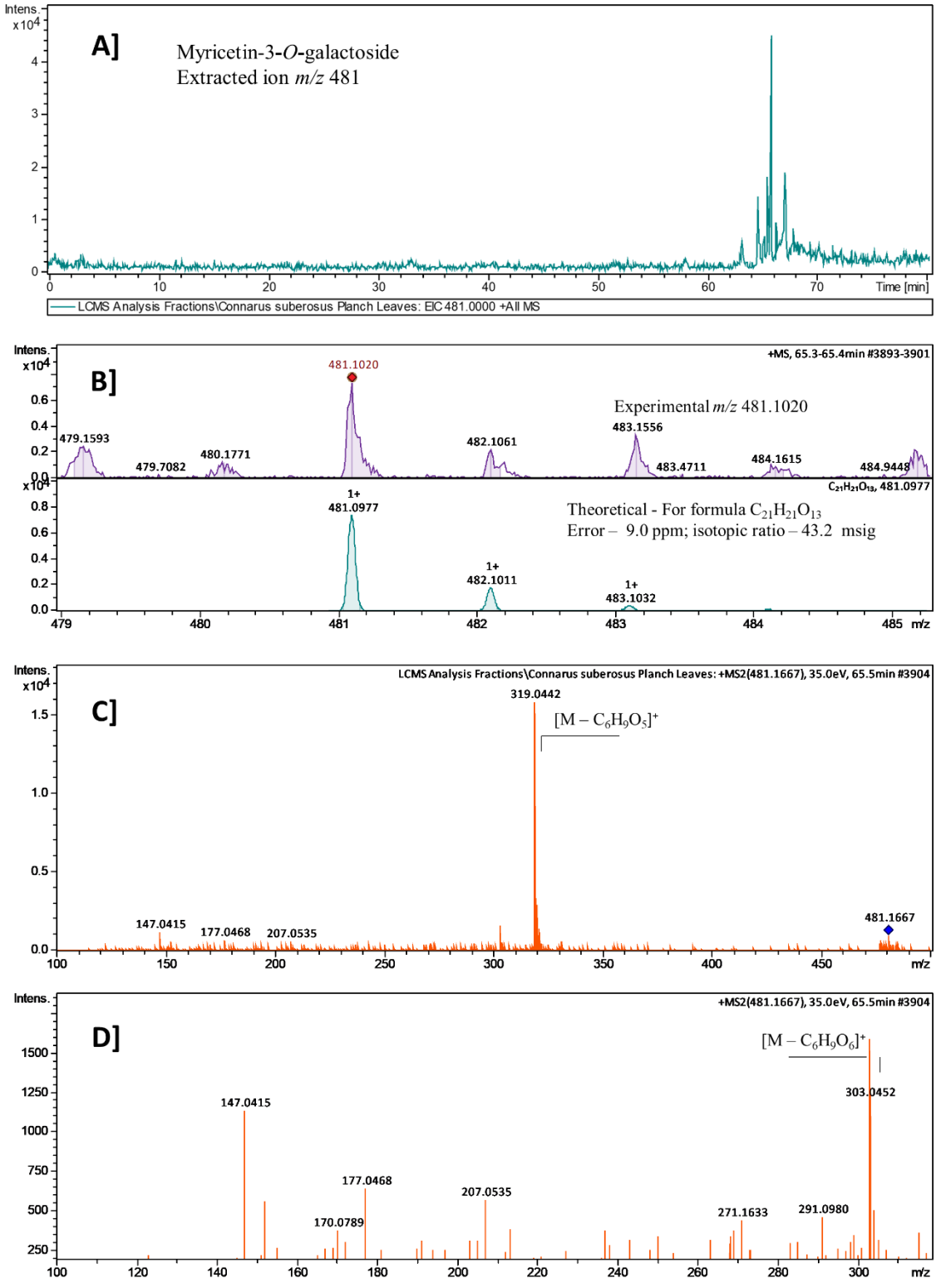


Figure 19 Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 465.1019 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.



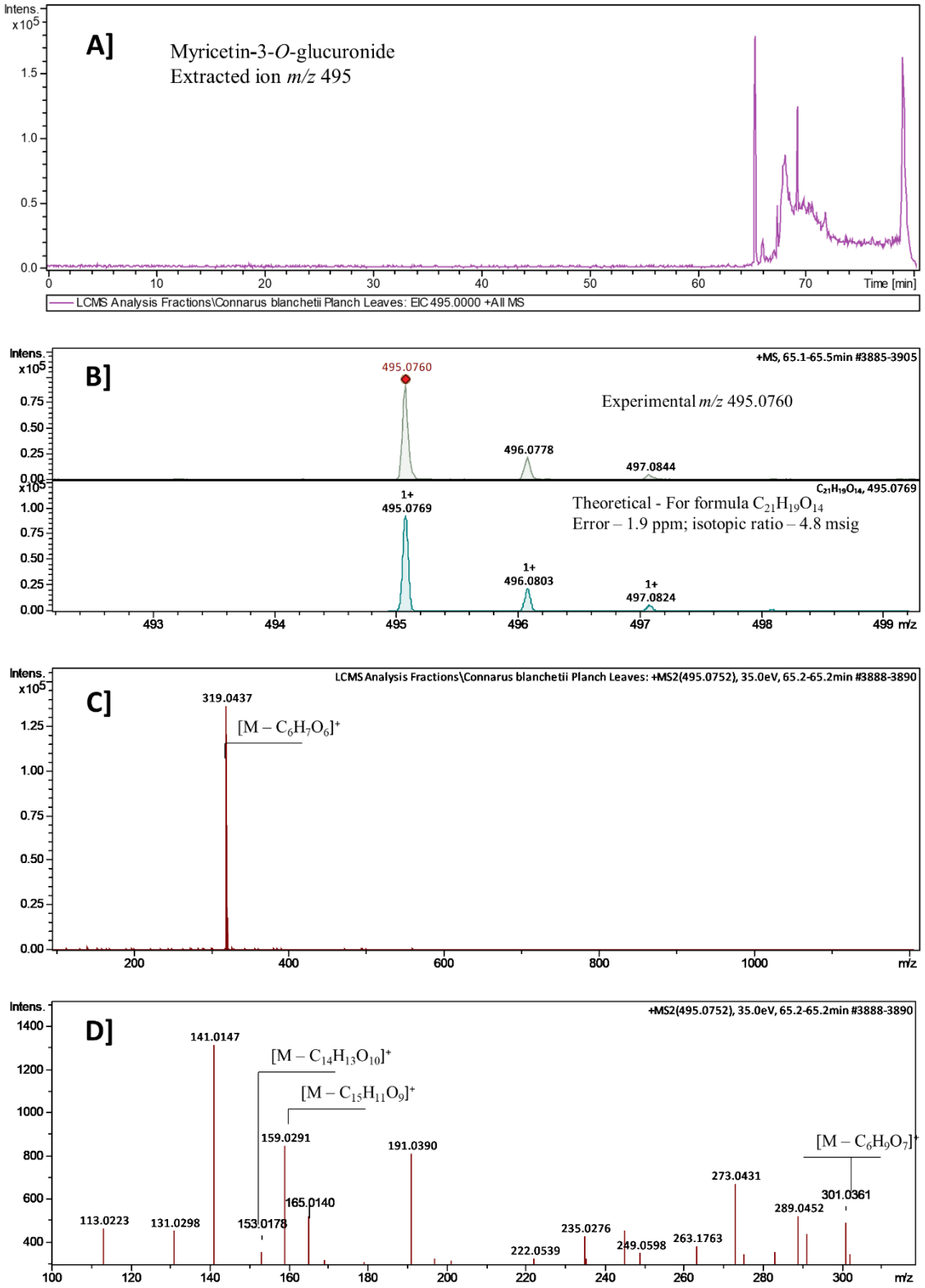
121

122 Figure 20: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
123 which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 479.0801
124 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID
125 and search for isotopic masses at ChemCalc.



126

127 Figure 21: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
128 which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 481.1020
129 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID
130 and search for isotopic masses at ChemCalc.



131

132 Figure 22: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
133 which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 495.0760
134 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID
135 and search for isotopic masses at ChemCalc.

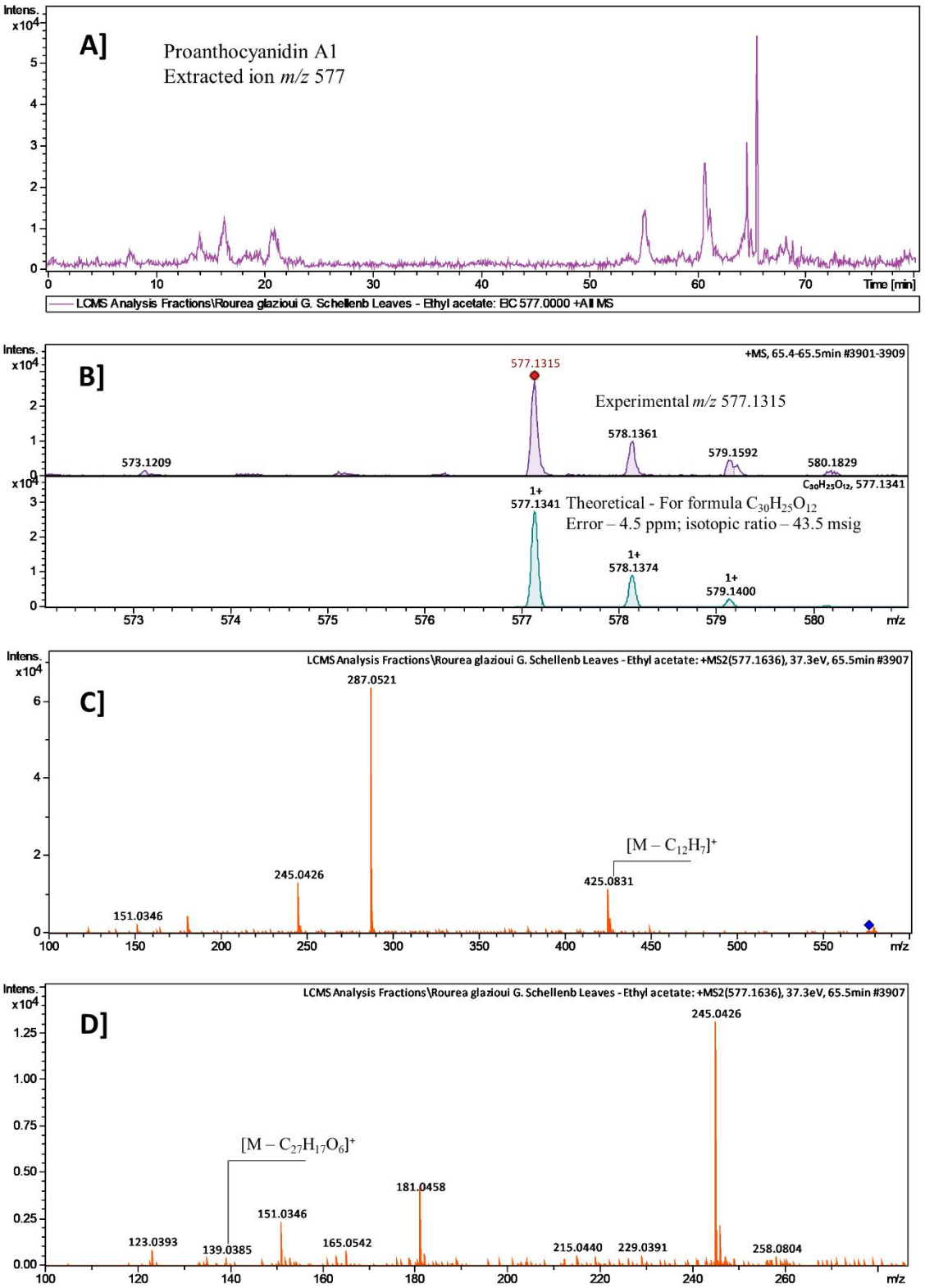


Figure 23: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 577.1315 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.

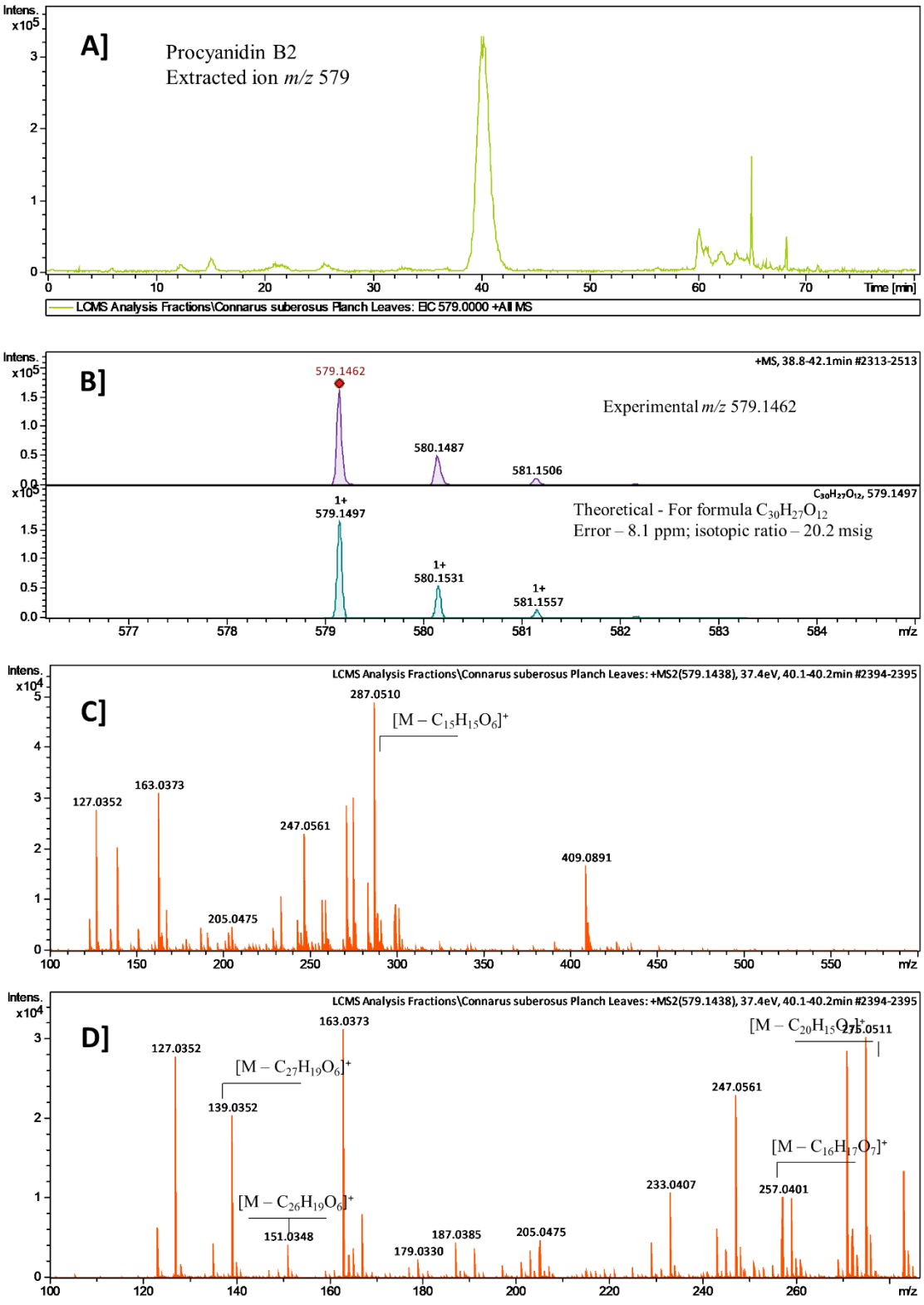
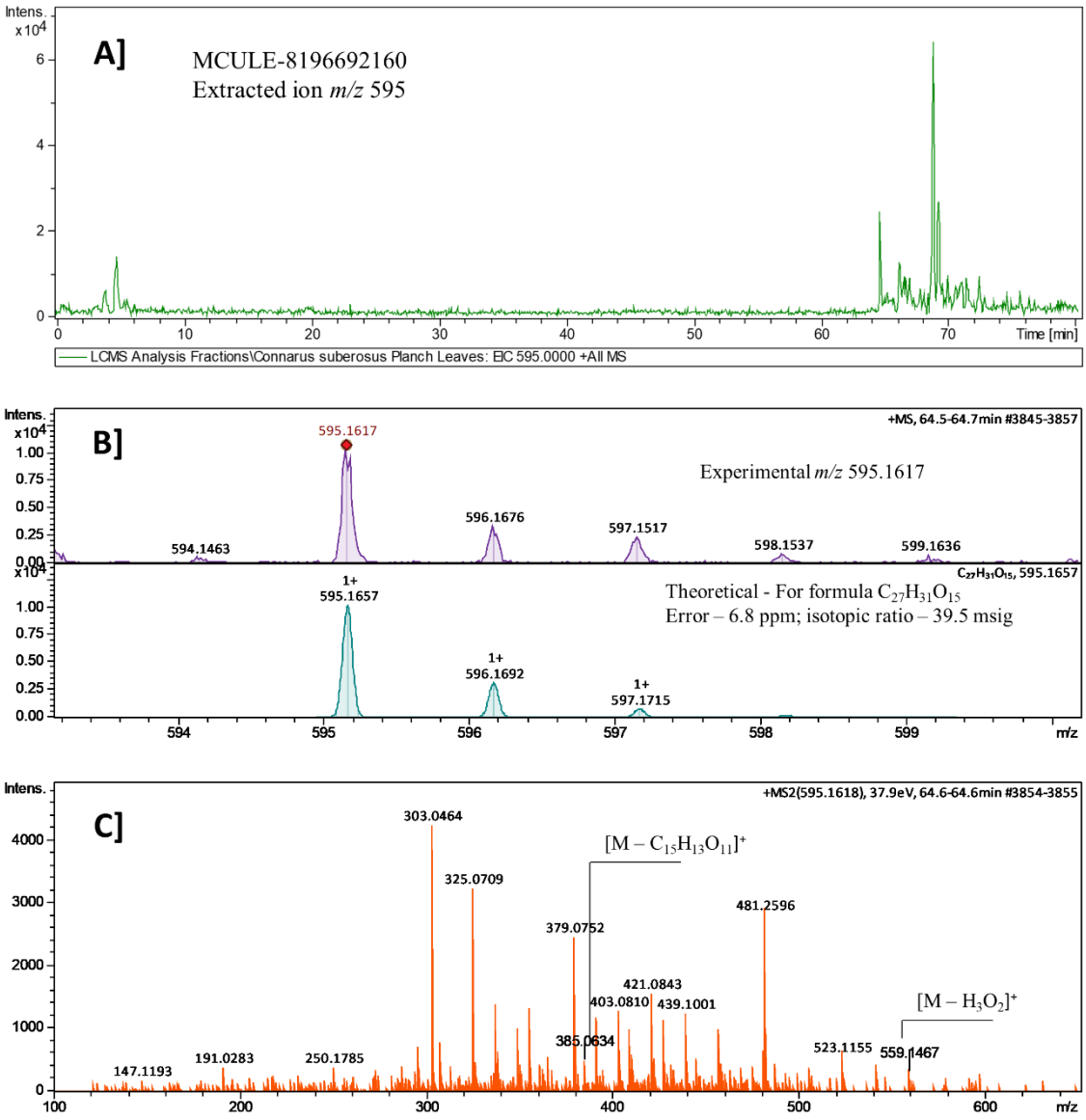
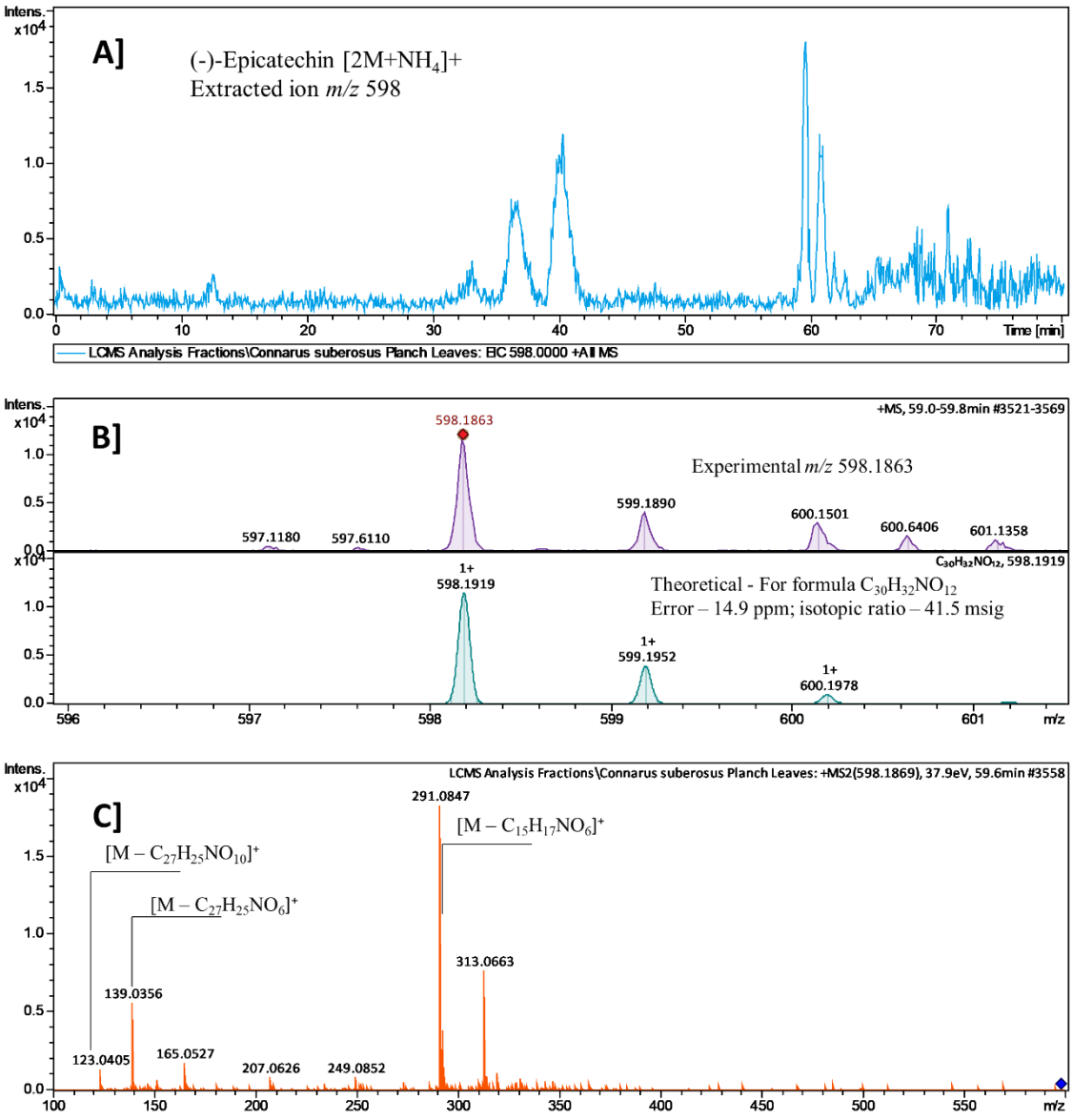


Figure 24: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 579.1462 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and search for isotopic masses at ChemCalc.



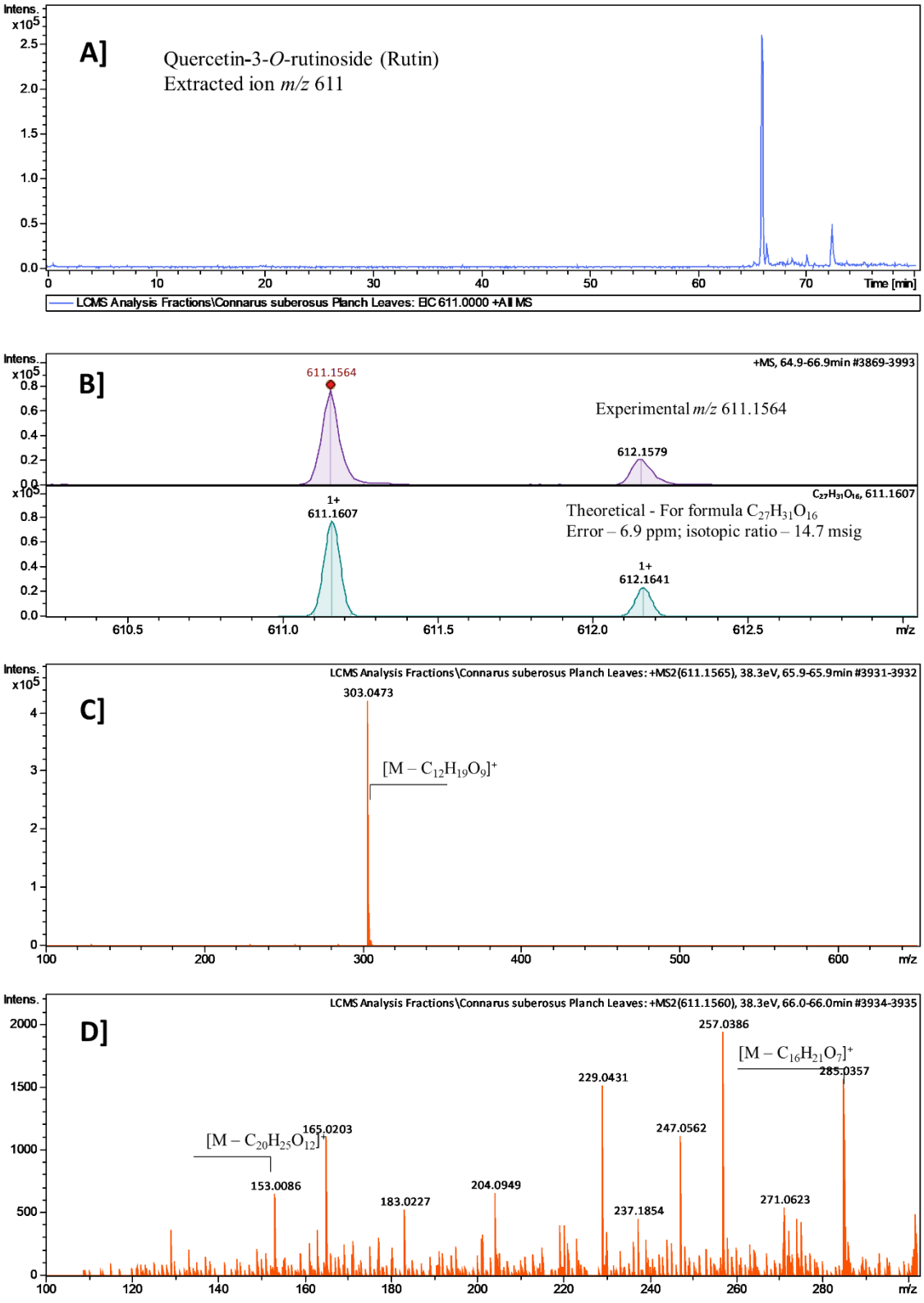
146

147 Figure 25 Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
148 which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 595.1617
149 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID
150 and search for isotopic masses at ChemCalc.



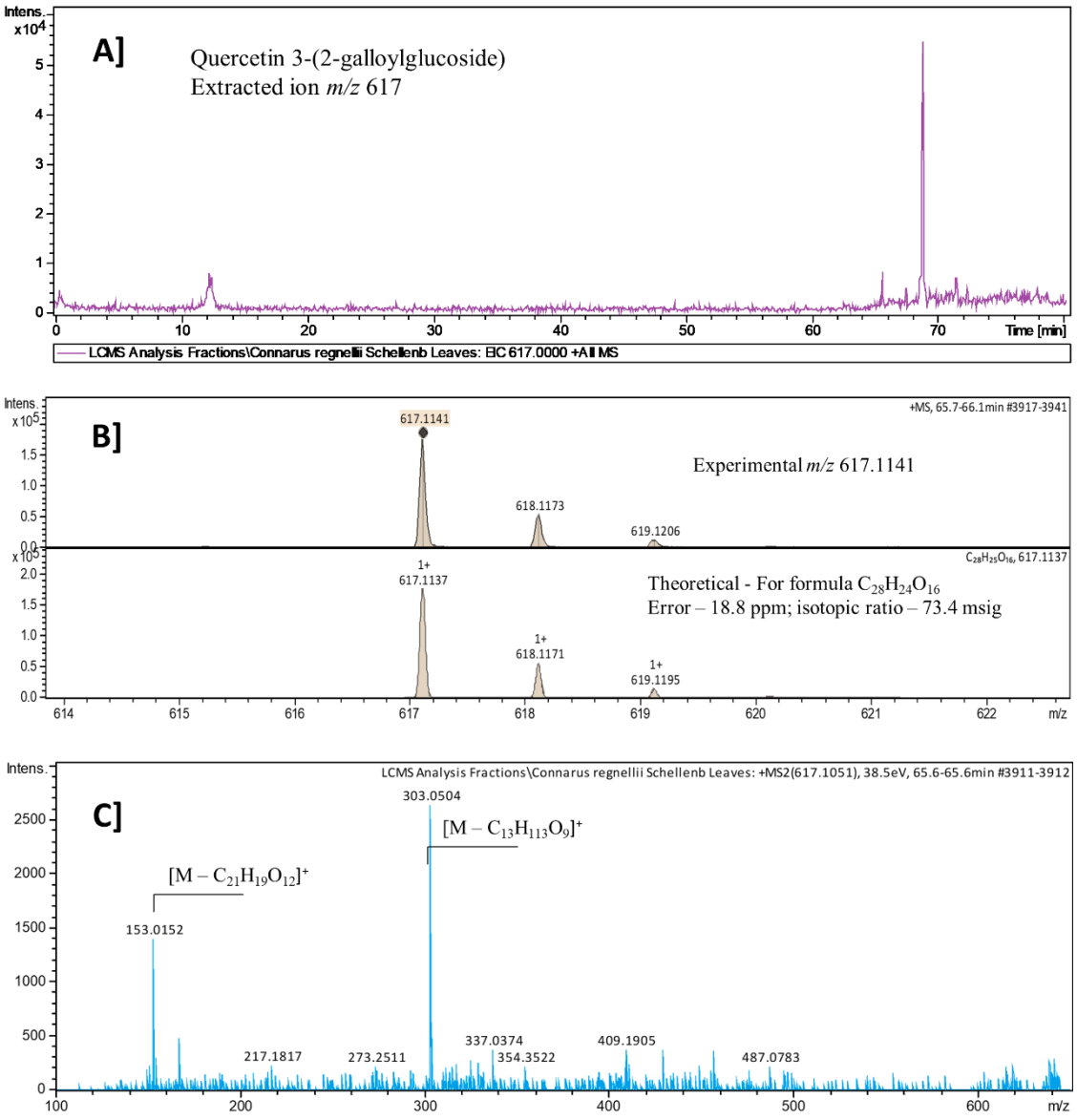
151

152 Figure 26: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
153 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 598.1863 in MS-
154 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
155 search for isotopic masses at ChemCalc.



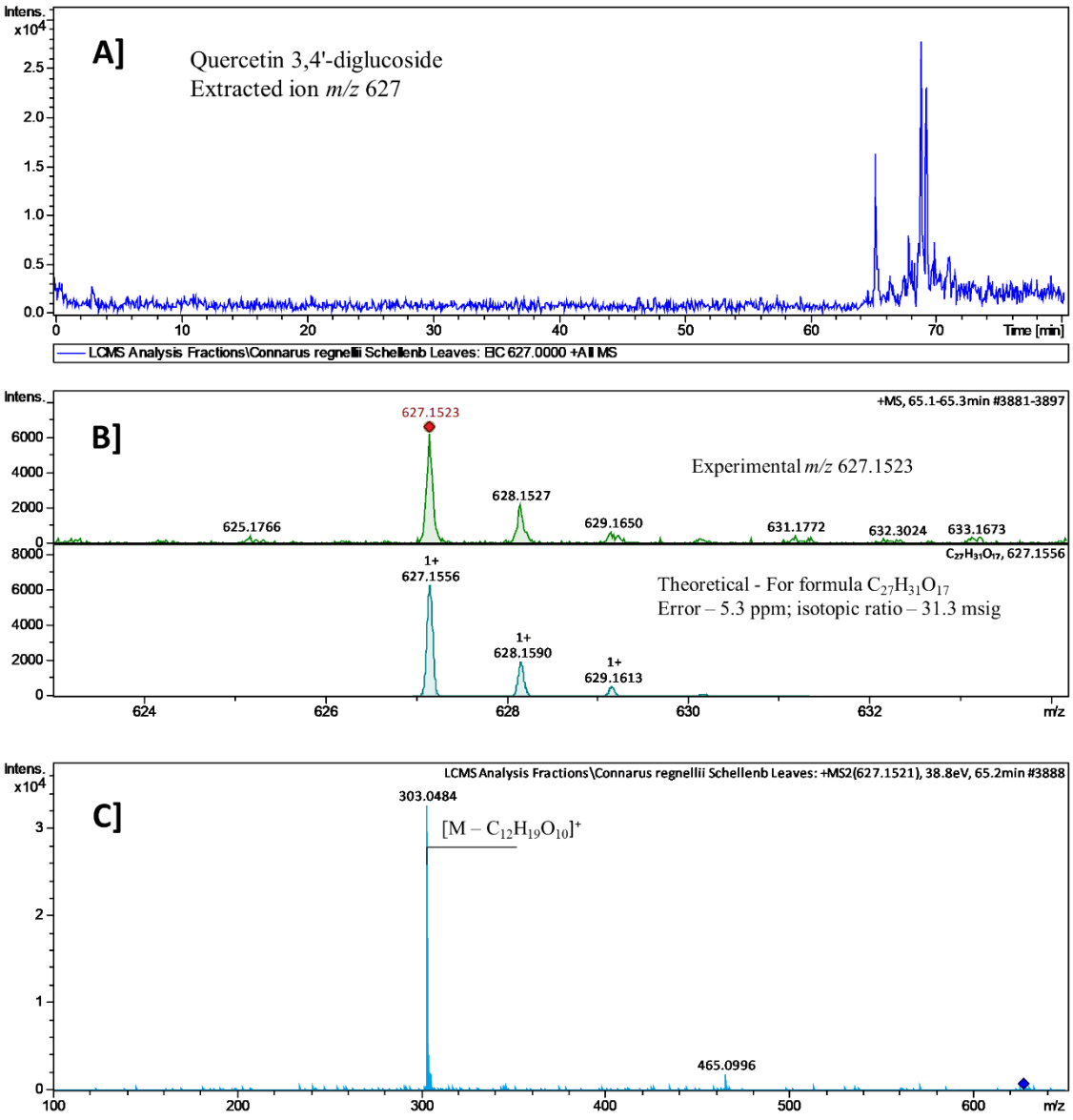
156

157 Figure 27: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
158 which was used for mass exact and isotopic ratio confirmation. In C) and D) analysis of ion m/z 611.1564
159 in MS-MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID
160 and search for isotopic masses at ChemCalc.



161

162 Figure 28: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
163 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 617.1141 in MS-
164 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
165 search for isotopic masses at ChemCalc.



166

167 Figure 29: Extracted ion chromatogram (HRMS) in A) Positive mode. In B) an expansion of full spectrum
168 which was used for mass exact and isotopic ratio confirmation. In C) analysis of ion m/z 627.1523 in MS-
169 MS mode HRESIMS spectrum black lines indicate peaks in the spectrum confirmed with CFM-ID and
170 search for isotopic masses at ChemCalc.

Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão

Cadastro nº A1EC16C

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**A1EC16C**
Usuário:	**LUIS FERNANDO NUNES ALVES PAIM**
CPF/CNPJ:	**009.834.430-76**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Rourea cuspidata

Título da Atividade:	**Isolamento e caracterização dos compostos químicos e estudo das atividades biológicas de Rourea cuspidata Benth. ex Baker**

Equipe

00983443076	**Universidade de Caxias do Sul**
Sidnei Moura e Silva	**Universidade de Caxias do Sul**

Data do Cadastro:	**18/04/2018 14:05:14**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
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Certidão

Cadastro nº A1FE9E7

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**A1FE9E7**
Usuário:	**LUIS FERNANDO NUNES ALVES PAIM**
CPF/CNPJ:	**009.834.430-76**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Rourea induta

Connarus suberosus

Rourea glazioui

Connarus blanchetii

Título da Atividade: **Isolamento e caracterização de compostos químicos com estudos de atividades biológicas de espécies vegetais da família Connaraceae.**

Equipe

LUIS FERNANDO NUNES ALVES PAIM	**Universidade de Caxias do Sul**
Joicelene Regina Lima da Paz	**Universidade de Brasília UnB-DF**
Cássio Augusto Patrocinio Toledo	**Universidade de Campinas**

Parceiras Nacionais

00.038.174/0001-43 / Fundação Universidade de Brasília

Data do Cadastro:	**03/12/2018 10:04:10**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
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SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN

Ministério do Meio Ambiente
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SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão

Cadastro nº A7E69F6

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**A7E69F6**
Usuário:	**Universidade de Caxias do Sul**
CPF/CNPJ:	**88.648.761/0001-03**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Rourea induta

Connarus suberosus

Rourea glazioui

Connarus blanchetii

Connarus regnellii

Título da Atividade: **Isolamento e caracterização de compostos químicos com estudos de atividades biológicas de espécies vegetais da família Connaraceae.**

Equipe

LUIS FERNANDO NUNES ALVES PAIM	**Universidade de Caxias do Sul**
Joicelene Regina Lima da Paz	**Universidade de Brasília UnB-DF**
Cássio Augusto Patrocinio Toledo	**Universidade de Campinas**

Parceiras Nacionais

00.038.174/0001-43 / Fundação Universidade de Brasília

Data do Cadastro:	**19/03/2019 18:48:51**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **16:26** de **27/08/2019**.

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Ministério do Meio Ambiente
CONSELHO DE GESTÃO DO PATRIMÔNIO GENÉTICO

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO

Certidão

Cadastro nº A251027

Declaramos, nos termos do art. 41 do Decreto nº 8.772/2016, que o cadastro de acesso ao patrimônio genético ou conhecimento tradicional associado, abaixo identificado e resumido, no Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado foi submetido ao procedimento administrativo de verificação e não foi objeto de requerimentos admitidos de verificação de indícios de irregularidades ou, caso tenha sido, o requerimento de verificação não foi acatado pelo CGen.

Número do cadastro:	**A251027**
Usuário:	**Universidade de Caxias do Sul**
CPF/CNPJ:	**88.648.761/0001-03**
Objeto do Acesso:	**Patrimônio Genético**
Finalidade do Acesso:	**Pesquisa**

Espécie

Rourea induta

Connarus suberosus

Título da Atividade:	**Isolamento e caracterização de compostos químicos com estudos de atividades biológicas de espécies vegetais da família Connaraceae.**

Equipe

LUIS FERNANDO NUNES ALVES PAIM	**Universidade de Caxias do Sul**
Joicelene Regina Lima da Paz	**Universidade de Brasília UnB-DF**

Parceiras Nacionais

00.038.174/0001-43 / Fundação Universidade de Brasília

Data do Cadastro:	**01/09/2018 17:07:29**
Situação do Cadastro:	**Concluído**

Conselho de Gestão do Patrimônio Genético
Situação cadastral conforme consulta ao SisGen em **16:25** de **27/08/2019**.

SISTEMA NACIONAL DE GESTÃO DO PATRIMÔNIO GENÉTICO E DO CONHECIMENTO TRADICIONAL ASSOCIADO - SISGEN